Lentiviral-mediated gene delivery to investigate the functional role of neuropsychiatric genes

Fernandes, Alinda

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Author: Alinda Fernandes

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Alinda Rose Fernandes

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This thesis is dedicated to my parents, Albert and Dorothy.

Abstract

Genetic studies have led to the identification of several candidate genes, some novel and others established, that may contribute to the risk of developing neuropsychiatric disorders.
For example, dopamine receptor genes are established candidates for a number of psychiatric disorders such as Parkinson's Disease, alcohol addiction and mood disorders. On the other hand, a gene of unknown function, *AUTS2* (Autism susceptibility candidate 2), has recently been associated with alcohol consumption in a GWAS meta-analysis performed by our group. Interestingly, it has been associated with a broad range of neuropsychiatric disorders including autism, epilepsy and schizo-affective disorders. This thesis looked to address two broad aims: to establish lentiviral-mediated gene delivery technique *in vivo* by delineating the role of two well characterised Dopamine receptors D2R and D3R and to functionally characterise the role of *AUTS2*. By successfully establishing lentiviral mediated gene manipulation *in vitro* and *in vivo*, this thesis presents data for a similar role of nucleus accumbens D2R and D3R in novelty-induced locomotion while these receptors have a differential function in the regulation of light-induced locomotor behaviour in rats. Additionally, using molecular biology and *in silico* methods, this thesis demonstrates that AUTS2 is a nuclear protein and presents indications of its function as a neurodevelopmental gene with a potential role in neural migration, although its specific role has yet to be corroborated. Collectively, findings from this thesis will increase our understanding of the genetic link with brain function and behavioural traits. This will therefore have implications for overall neuropsychiatric research, as it will help understand molecular mechanisms underlying these conditions and possibly direct in the identification of potential therapeutic targets.

Brief summary of thesis chapters

Chapter One provides an overview of current literature concerned with the use of various strategies used in the identification and validation of candidate genes involved in the aetiology of neuropsychiatric disorders in general.

Chapter Two describes generalised methods and materials used in the data chapters that follow.

Chapter Three presents findings from establishing lentiviral mediated gene delivery *in vitro* and *in vivo* by targeting two Dopamine receptors D2 and D3 in the rat nucleus accumbens to distinguish their roles on locomotor function.

Chapter Four presents findings that contribute to the regional and cellular characterisation of *AUTS2* (Autism susceptibility candidate 2) whose gene function is largely unknown.

Chapter Five attempts to investigate the neurodevelopmental role for *AUTS2* using lentiviral mediated gene silencing in a human foetal neural stem cell line, given its characterisation as a neurodevelopmental gene and its implications in a broad range of neuropsychiatric disorders.

Chapter Six will discuss the above findings and its implications for scientific research. This chapter will also discuss limitations of the series of research presented in this thesis.

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Finally, but most importantly, I am eternally grateful to my mum and dad for their motivation and constant encouragement throughout my life and my siblings, Andrea and Adrian for just being there.

Statement of independent work

Chapter 1

This chapter is entirely my own work.

Chapter 2

This chapter is entirely my own work.

Chapter 3

My contribution included the generation of plasmids overexpressing and silencing D2R and D3R, as well as lentiviral production, ultacentrifugation, quantification and testing expression/ silencing efficiency of these constrcuts *in vitro*. I have also performed all the histological analysis in this chapter. Animal handling, behavioural testing, culling and brain extraction which were carried out by Dr Alanna Easton.

Chapter 4

My contribution involved the generation of plasmids overexpressing AUTS2 and V5-tagged AUTS2, tranfecting these plasmids in various cell lines as well as carrying out RNA and protein analysis post-transfection. For co-immunoprecipitation of AUTS2, I was assisted by Dr David Stacey.

Chapter 5

My contribution included the production, ultracentrifugation, quantification of AUTS2-sielcning lentiviruses. In addition, I performed the transduction of 293T cells to assess knock down efficiencies of lentiviruses silencing AUTS2, as well as carried out RNA extraction for microarrays. Microarray experiments were performed by Eric Nasser, Biomedical Research Centre (BRC) genomics facility, King's College London. The data analysis was done by Dr Venu Pullabhatla, NIHR GSTFT/KCL Comprehensive Biomedical Research Centre.

Chapter 6

This chapter is entirely my own work.

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List of Abbreviations

°C	Degrees Centigrade
ΔCt	Delta Cycle threshold
µg	Micrograms
µl	Microlitres
µm	Micrometres
µM	Micromolar
ADHD	Attention Deficit Hyperactivity Disorder
4-OHT	4-hydroxy-tamoxifen
ASD	Autism Spectrum Disorders
ATCC	American Type Culture Collection
ATRA	All-trans retinoic acid
AUTS2/ Auts2	Autism susceptibility candidate 2
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
chr	Chromosome
$CaCl_2$	Calcium chloride
c-MYC	V-myc avian myelocytomatosis viral oncogene homolog
CNV	Copy Number Variant
Co-IP	Co-immunoprecipitation
Ct	Cycle threshold
DA	Dopamine
DR	Dopamine receptor
D2R	Dopamine D2 receptor
D3R	Dopamine D3 receptor
DAPI	4'-6-Diamidino-2-phenylindole
DAPT	3,5-**D**ifluorophenacetyl)-L-**a**lanyl]-S-**p**henylglycine **t**-butyl ester
dATP	DeoxyAdenosine-5'-triphosphate
d.f.	Degrees of Freedom
dH_2O	Distilled water (molecular biology grade)
ddH_2O	Double Distilled water

DMEM	Dubelcco's modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded deoxyribonucleic acids
EBI	European Bioinformatics Institute
ECACC	European Collection of Cell Cultures
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
fmols	Femtomole (10^{-15} moles)
pmoles	Picomoles (10^{-9} moles)
γ	Gamma
g	Grams
g	Gravity
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Florescent Protein
GWAS	Genome-wide association studies
h	hour
HBS	Hepes Buffer Saline
HCl	Hydrogen chloride
ID	Identification
kb	Kilobases
kDa	KiloDaltons
LTR	Long terminal repeat
M	Molar
MCS	Multiple cloning site
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
MR	Mental retradation
mRNA	Messenger RNA
MZ	Monozygotic twins

n	Number
NAcc	Nucleus Accumbens
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NeuN	Neuronal nuclei
ng	Nanograms
NP-40	Nonyl phenoxypolyethoxylethanol
NSCs	Neural stem cells
nt	nucleotide
OD	Optical density
OMIM	Online Mendelian Inheritance in Man
ONS	Office for National Statistics
OR	Odds ratio
ORF	Open Reading Frame
p	Short arm of chromosome
PPI	Protein-protein interaction
PBS	Phosphate buffered saline
PBST	PBS-Triton
PCR	Polymerase chain reaction
q	Long arm of chromosome
qPCR	Quantitative polymerase chain reaction (RT-PCR)
rcf	Relative centrifugal force
ref	Reference
RIPA buffer	Radioimmunoprecipitation Assay Buffer
RMM+	Reduced Modified Medium with growth factors
RMM-	Reduced Modified Medium without growth factors
RNA	Ribonucleic acids
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Real Time - PCR
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis

SEM	Standard error mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
Taq	Thermus aquaticus polymerase
TBS	Tris buffered Saline
TBST	TBS-Tween
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
U	Units
UCSC	University of California, Santa Cruz
UK	United Kingdom
UniProt	Universal Protein Resource
USA	United States of America
V	Volts (voltage)
v/v	Volume per volume
w/v	Weight per volume

Chapter 1 Introduction

The field of psychiatric genetics is rapidly expanding since the discovery that behaviour and psychiatric traits are, to some extent, explained by genetic influences, in addition to environmental factors. Gene identification methods, including candidate gene studies, linkage studies, and more recently, genome wide association studies are currently proving to be key contributors to the identification of novel gene and gene variants associated with several neuropsychiatric disorders. These studies have also revealed that a wide range of neuropsychiatric disorders have overlapping genetic aetiology with many of the genes involved in neurodevelopment. Subsequent functional genetic research complements findings from linkage and association studies by elucidating the molecular mechanisms by which these genes exert their effect on behaviour and psychiatric traits. Gaining insight into the underlying genetic basis of neuropsychiatric disorders and mechanisms through which genes exert their effects on behaviour will contribute significantly to the development of efficient prevention strategies and providing personalized treatments. It is thus imperative to identify susceptibility genes and elucidate the mechanisms by which genetic variation alters molecular function, thus predisposing to psychiatric disorders. This thesis is split into two themes: it investigates the role of two dopamine receptors (D2R and D3R) in locomotor behaviour by establishing lentiviral mediated gene delivery technique; and functionally characterise the role of AUTS2, a gene of unknown function associated with various neuropsychiatric disorders including autism, depression, epilepsy and schizo-affective disorders. This chapter will provide an overview of the approaches and techniques used in gene identification and gene function studies relevant to neuropsychiatric disorders in general and conclude with the overall aims of this thesis.

1.1 The prevalence of neuropsychiatric disorders

The term neuropsychiatric disorders encompasses a broad range of mental disorders including substance abuse and dependence, schizophrenia, depression and other mood disorders, as well as neurological diseases like dementia, autism and epilepsy. These diseases present a large burden on families and society. In the EU alone, the three major contributors to the overall disease burden in 2010, were depression (7.2%), dementia (3.7%) and alcohol abuse (3.4%) (Wittchen *et al.*, 2011). The overall disease burden is mainly due to disability, resulting in the inability to carry out daily routines, making neuropsychiatric disorders accountable for work disability and economic loss.

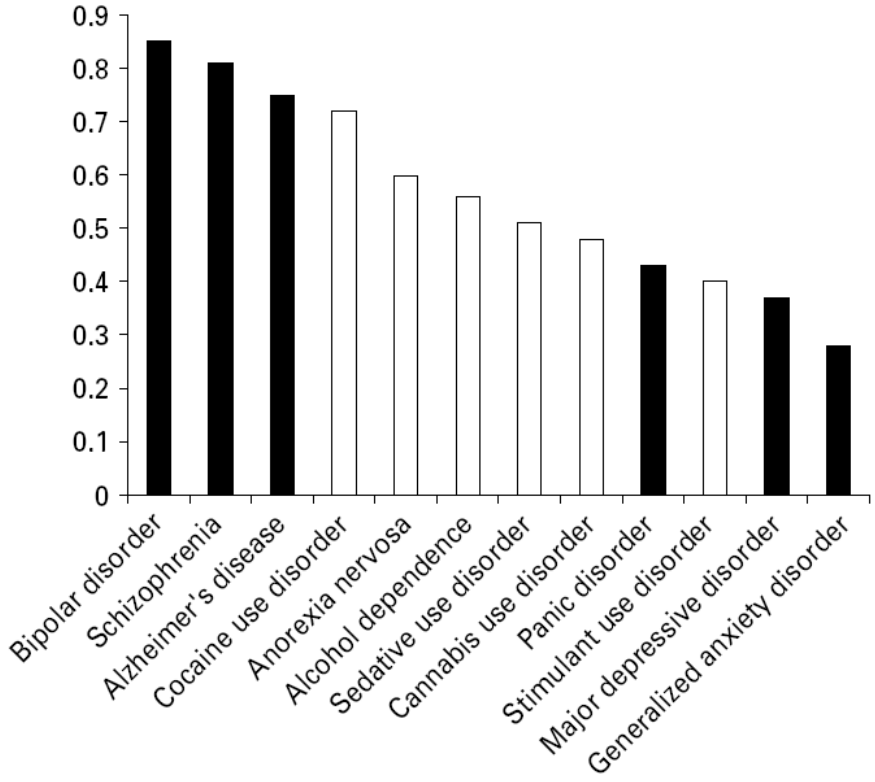
1.2 Genetic contribution to neuropsychiatric disorders

It has been extensively documented that genetic factors strongly influence the aetiology of neuropsychiatric disorders. Family, twin and adoption studies have provided evidence of genetic predisposition to many neuropsychiatric disorders with variation in the extent of genetic influence across disorders as depicted in Figure 1. Depression and anxiety are reported to be least heritable i.e. <40% (Hettema *et al.*, 2001; Bienvenu *et al.*, 2011), while heritability estimates for alcohol abuse and dependence is moderate ranging from 30% to 60% (Agrawal *et al.*, 2008). Schizophrenia, bipolar and autism spectrum disorders are highly heritable with estimates of 80% or more (Muhle *et al.*, 2004; Bienvenu *et al.*, 2011) indicating that the major underlying susceptibility of these disorders are genetic. These findings also show that disease susceptibility is also influenced by environmental factors as concordance rates in monozygotic twins do not reach 100%. In fact, environmental risk factors have been examined for interaction with specific genes in some neuropsychiatric conditions (Caspi *et al.*, 2003; Lesch, 2004; Bukh *et al.*, 2009; Clarke & Schumann, 2009) establishing that environmental triggers such as stress and childhood abuse are important determinants of disease susceptibility in genetically predisposed individuals.

It is now widely agreed that psychiatric disorders are of multifactorial aetiology, with several genetic and environmental factors contributing to disease development. Determinants of complexity are likely to include genetic heterogeneity, polygenicity, phenocopies, gene vs environment interaction and incomplete penetrance. Despite the intricate genetic heterogeneity of neuropsychiatric disorders, a number of gene identification approaches have been successful in uncovering susceptibility genes as described in the following subsections.

Figure 1: Heritability estimates for psychiatric disorders or traits (black) and behavioural disorders (white).

This figure was taken from (Bienvenu et al., 2011). Bipolar disorder, Schizophrenia and Alzheimer's disease have the highest heritability, compared to substance use disorders which are moderately heritable, while anxiety and major depressive are the least heritable from this list.



1.3 Gene identification approaches in neuropsychiatric disorders

Over the past few years, strategies aiming to identify genes that contribute to disease susceptibility have employed either linkage and association analysis or candidate gene approach. Briefly, linkage and association studies are two complementary approaches used to identify genetic loci or genes associated with a particular behavioural trait or disorder, usually in a hypothesis free fashion. On the other hand, candidate gene approach, involves the selection of genes known to influence processes in the pathogenesis or treatment of a disorder.

1.3.1 Linkage studies

This approach enables the identification of genomic regions that contain genes or genetic markers that predispose to diseases in families with multiple affected members, which occur more often than expected by chance. Markers that co-segregate with the disease indicate which genomic regions contain disease susceptibility genes. Using this hypothesis-neutral approach, relatively few genetic markers are necessary to distinguish which chromosome segments are shared among relatives with a common phenotype. Most linkage studies are performed in a collection of many families, each with few affected individuals. For linkage analysis to succeed, markers that flank the disease gene must segregate with the disease in families, hence this method is largely successful with diseases following monogenic Mendelian inheritance. Large numbers of samples are required as this approach is not very powerful for the detection of susceptibility loci with a small contribution to the total disease risk which is the case for many neuropsychiatric disorders (Risch & Merikangas, 1996). Nonetheless, this method has been successful in identifying susceptibility genes for non-psychiatric disorders, such as evidence for linkage to chromosomal regions 6p, 8p and 13q has been consistently reported for schizophrenia (Gill *et al.*, 2010). Extensive fine mapping of these regions led to the discovery of three susceptibility genes: the dysbindin gene at 6p (*DTNBP1*), *NRG1* at 8p and protein G72 at 13q, all of which linkage have been confirmed by independent studies (Maier *et al.*, 2003; Benson *et al.*, 2004).

1.3.2 Candidate gene approach

Genetic association studies on candidate genes have been a popular alternative to genetic linkage studies because families with multiple affected individuals are not necessary for this approach. These studies are based on a priori knowledge concerning the pathophysiology of the disease or phenotype in question. Not surprisingly, particular emphasis has been directed towards studying genes involved in the serotonergic and dopaminergic mechanisms, pivotal in neurobiological function regulating several emotional, cognitive, motor and reward processes. Hence, mood and personality disorders, anxiety, psychosis, substance abuse among other psychiatric conditions have been associated with dysfunction of these systems.

The serotonin transporter *SLC6A4* is a promising candidate gene for its involvement in a variety of psychiatric disorders and it is also a target for drugs (serotonin selective reuptake inhibitors) that are used for the treatment of mood and anxiety disorders. The long and short allele of the

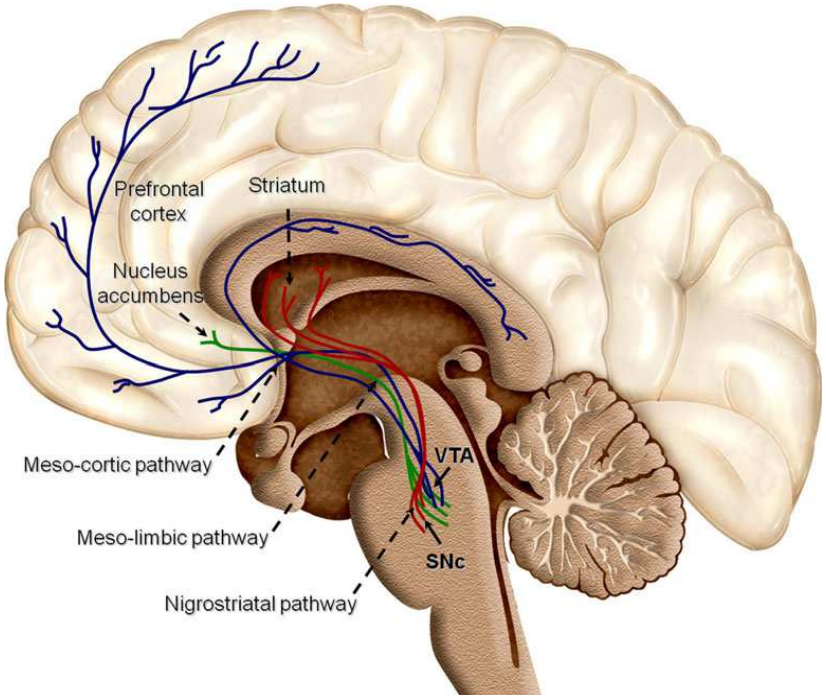
serotonin transporter results from a polymorphism in the promoter region of the serotonin transporter gene (*5-HTTLPR*; 5-hydroxytryptophan transporter linked polymorphic region) which was shown to be associated with different levels of gene expression (Heils *et al.*, 1996). The alleles are caused by a 44bp insertion/deletion approximately 1kb upstream of the *SLC6A4* gene; homozygosity of the short allele results in lower mRNA and protein expression (Lesch *et al.*, 1996). Numerous studies have reported an association of this polymorphism with several psychiatric disorders (Lazary; Caspi *et al.*, 2003; Feinn *et al.*, 2005; Roy *et al.*, 2007). Among the 14 serotonin receptor subtypes, a substantial portion of studies have looked at association of *5HTR1A* and *5HTR2A* polymorphisms and mood disorders and schizophrenia (Verga *et al.*, 1997; Spurlock *et al.*, 1998; Tsai *et al.*, 1999; Oswald *et al.*, 2003; Abdolmaleky *et al.*, 2004; Hettema *et al.*, 2008; Kishi *et al.*, 2011). Another serotonergic component shown to be relevant for depression in particular is the monoamine oxydase (MAO) enzyme that catabolises the serotonin neurotransmitter. There are two forms *MAO-A* and *MAO-B*, of which it has been suggested that *MAO-A* has higher activity (Shih & Chen, 1999). Accordingly, *MAO-A* gene polymorphisms and SNPs have been identified as possible risk factors for major depression and bipolar disorder (Schulze *et al.*, 2000; Caspi *et al.*, 2002; Du *et al.*, 2004; Muller *et al.*, 2007b).

Likewise, the dopaminergic system is known to be essential in the regulation of several behavioural functions including locomotion, feeding, sleep, attention, cognition, emotion as well as neuroendocrine functions (Lindvall *et al.*, 1983). This system comprises of four major neuronal pathways. One of these is the nigrostriatal pathway which consists of the dopaminergic neurons in the substantia nigra neurons which project to the striatum and play a prominent role in controlling motor function (Figure 2). Accordingly, dysfunction in this pathway principally results in Parkinson's disease (Strange, 1993b). Other pathways are the mesocortical and mesolimbic pathways (also called mesocorticolimbic pathway) connect the neurons of the ventral tegmental area (VTA) to the cortex and the limbic system. The mesolimbic system includes dopaminergic innervations in the VTA that project mainly to the NAcc as well as the olfactory tubercle. While the mesocortical dopaminergic system also includes the VTA, it extends its fibres in the prefrontal cortex, cingulate and peririhinal cortex (Wise, 2004; 2005). These pathways regulate cognitive functions, emotion and reward (Yim & Mogenson, 1980; D'Ardenne *et al.*, 2008; Phillips *et al.*, 2008). It has been reported that alterations in the mesocorticolimbic pathway contribute to the development of psychosis (Liston *et al.*; Kapur & Mamo, 2003; Phillips *et al.*, 2003; Seeman *et al.*, 2006; Laviolette, 2007), ADHD (Iversen & Iversen, 2007), and addiction (Kalivas & Volkow, 2005; Koob & Le Moal, 2008)

among many other psychiatric conditions. Lastly, the tuberoinfundibular pathway regulates prolactin secretion from the anterior pituitary gland through dopamine released from a population of neurons that are present in the 'tuberal region' of the hypothalamus.

Figure 2: The dopaminergic neural circuitry in the brain

This figure taken from (Arias-Carrion et al., 2010), depicts the three main dopaminergic pathways through which the neurotransmitter dopamine exerts its effect on various psychomotor behaviours: mesocortical, mesolimbic and nigrostriatal pathways.



Due to the multiple pathways it is involved in, it is not surprising that the dopamine hypothesis of schizophrenia and psychosis prompted a large number of studies to focus on association of genes involved in the dopaminergic system and psychosis (Ni *et al.*, 2002; Severino *et al.*, 2005; Dmitrzak-Weglarz *et al.*, 2006; Del Zompo *et al.*, 2007). This model attributes symptoms of schizophrenia and other psychosis to hyperactive dopaminergic signalling originating from observations of dopamine blocking effects of antipsychotic drugs (Moncrieff, 2009). Additionally, due to its involvement in the reward pathway, a substantial portion of studies have aimed to reveal associations between dopamine receptors and alcohol addiction. There are 5 different isoforms for the dopamine receptors: *DRD1*, *D2D2*, *DRD3*, *DRD4*, and *DRD5*.

One of the first candidate gene studies in alcoholism was the dopamine receptor gene *DRD2* with its *Taq*1-A1 polymorphism (Blum *et al.*, 1990; Parsian *et al.*, 1991; Noble, 2000). *DRD4* is also implicated in a recent study (Hack *et al.*, 2011) while the same study also reported that *DRD3* and *DRD5* are typically not associated with alcohol dependence, indicating that receptors encoded by these genes may not be associated with alcohol dependence. Other genes in the dopaminergic system have also been implicated as candidate genes such as the dopamine transporter (DAT), encoded by the *SLC6A3* gene which initiates the reuptake of extracellular synaptic dopamine by the pre-synaptic neurons, thus regulating dopaminergic signalling (Alves *et al.*, 2011). The most widely studied variant in *SLC6A3* has been the variable number tandem repeat (VNTR) region located in the 3' UTR, with alleles having between 3-16 repeats (van der Zwaluw *et al.*, 2009).

Notably, the most robust of all allelic variations relevant for psychiatric disorders is the association between the genes encoding hepatic enzymes alcohol dehydrogenase (ADH; coverts alcohol to acetaldehyde) and aldehyde dehydrogenase (ALDH; converts acetaldehyde to acetate). Indeed, these are excellent candidate genes as a priori knowledge indicates that they are clearly involved in regulating the levels of alcohol in the bloodstream and the amount of alcohol that reaches the brain. There are seven different *ADH* genes in the human genome that form a cluster on chromosome 4q (5'-*ADH7-ADH1C-ADH1B-ADH6-ADH4-ADH5*-3') and two main ALDH genes (*ALDH1A1* and *ALDH2*) (Chen *et al.*, 2009). Genetic variants that confer a protective effect on the development of alcoholism include a high activity isoform of ADH (referred to as *ADH1B*2*) leading to an accumulation of acetaldehyde in the blood consequently producing adverse effects like nausea, hypotension and headache. This polymorphism (His47Arg) conferring this protective effect is commonly found in population of Asian descent. Furthermore, linkage studies have shown that the *ADH* gene cluster is within a susceptibility region for alcohol-related phenotypes in European populations (Reich, 1996; Reich *et al.*, 1998; Cichon *et al.*, 2009). Several other independent linkage studies have replicated this finding using different populations such as Irish sibling pairs (Prescott *et al.*, 2006); and South Western Native American population (Long *et al.*, 1998). In fact, this gene is one of the most consistently replicated finding in both linkage and association studies. Although this approach is very useful, other approaches are clearly required to discover novel genes associated with a given disease; GWAS are currently the method of choice to achieve this.

1.3.3 Genome Wide Association Studies (GWAS)

GWAS offer the possibility to identify specific genes that influence the development of a given psychiatric disorder, using a hypothesis-free approach. It is based on comparing the genotypes of affected vs unaffected individuals in a population to find genetic variation associated with the disease or the trait. Unlike linkage studies, GWAS enable the detection of 'small risk' genetic variation. In just the last few years, GWAS have successfully revealed associations of common DNA variants (both previously established and novel) with over 80 diseases and traits including schizophrenia, bipolar, major depression, attention deficit disorder (ADD), autism and alcohol dependence (Hirschhorn & Daly, 2005; Cichon *et al.*, 2009). Recently, our group in collaboration with others have identified Autism susceptibility candidate 2, *AUTS2*, as a possible candidate gene for alcohol consumption.

Previous studies did not find particular gene(s) that consistently showed significant associations across studies (Nurnberger *et al.*, 2001; Schuckit *et al.*, 2001; Treutlein *et al.*, 2009; Bierut *et al.*, 2010; Edenberg *et al.*, 2010). A possible reason for this could be due to the varied ethnic backgrounds of the participants resulting in genetic heterogeneity as mentioned earlier. Also, the alcohol phenotype described may have differed among the studies. Additionally, the few GWAS findings reported have not been confirmed by functional studies. As previously suggested, a large meta-analysis would arguably provide enough power to detect SNPs significantly associated with alcohol dependence (Kimura & Higuchi, 2011). Indeed, our group has conducted a meta-analysis of GWAS on alcohol consumption based on more than 28,000 drinkers plus a replication sample of around 21,000 drinkers of European ancestry. Most notably, Autism susceptibility candidate 2, *AUTS2* attained genome wide significance overall (discovery and replication samples combined) $p = 4.2 \times 10^{-08}$ (Schumann *et al.*, 2011). In the same study, evidence from *Drosophila* functional studies in which the *AUTS2* homologue was silenced showed reduced alcohol sensitivity; though *Drosophila* studies have their limitations in interpreting this kind of behaviour. This study points to a possible role of *AUTS2* in alcohol consumption. Interestingly, this gene has been implicated in several other neuropsychiatric disorders as discussed in the following section.

1.4 Overlapping genetic aetiology of neuropsychiatric disorders and *AUTS2*

It is now clear that the underlying genetic causality for neuropsychiatric disorders can be explained by the effects of many different alleles at various loci, each conferring a small effect. Following on from this, is the emerging idea that there is substantial overlap in the genetic aetiology of some neuropsychiatric disorders with a small number of publications vindicating this concept until now. For example, molecular genetic studies have revealed that several large copy number variants (CNVs) substantially increase the risk of schizophrenia, autism spectrum disorders (ASDs), ADHD and epilepsy (Owen *et al.*, 2011). Additionally, genetic studies indicate that the chromosome 16p13.11 heterozygous deletion is associated with these disorders (Ramalingam *et al.*, 2011). Further supporting this idea is the existence of co-morbidity among psychiatric disorders might be caused to some extent by genetic overlap (Schulze *et al.*, 2012). Large-scale epidemiological studies have shown individual and familial comorbidity between schizophrenia, ASDs, epilepsy, bipolar disorder, major depression, ADHD and other psychiatric diagnoses (Qin *et al.*, 2005; Daniels *et al.*, 2008; Steinhausen *et al.*, 2009). Moreover, a recent large scale study has shown that relatives of individuals affected with schizophrenia have increased risks of bipolar disorder and vice versa (Lichtenstein *et al.*, 2009). Several other studies have pointed out the idea that there exists a fundamental aetiological genetic overlap between what have largely been defined clinically as distinct disorders (Rzhetsky *et al.*, 2007; Friedman *et al.*, 2008; Mefford *et al.*, 2008; Guilmatre *et al.*, 2009; Sebat *et al.*, 2009; Ching *et al.*, 2010). Further research exploring the overlapping genetic aetiologies of psychiatric disorders is no doubt warranted.

Recent findings on *AUTS2* association with a number of neuropsychiatric disorders have also supported the view of their overlapping genetic aetiology. *AUTS2* was initially identified as an autism susceptibility candidate gene in a pair of monozygotic twins (MZ) with autism who had identical balanced chromosomal translocation at t(7;20) (q11.2; p11.2) which disrupted this gene (Sultana *et al.*, 2002). Similarly, other studies have also identified *AUTS2* disruptions in patients with different severities of ASD and/or mental retardation (MR). For example, three unrelated patients with MR had *AUTS*2 truncated by a *de novo* balanced translocation of 7q11.2 (Kalscheuer *et al.*, 2007), while a similar *de novo* 7q inversion breakpoint disrupting *AUTS2* and *CNTNAP2 (*contactin associated protein-like 2) genes were discovered in a patient with ASD/MR (Bakkaloglu *et al.*, 2008). Recently, a patient with ASD was found to have a *de*

novo balanced translocation that disrupted *AUTS2* (Huang *et al.*, 2010b). These reports demonstrate that *AUTS2* plays a significant role in human brain function, especially the aetiology of ASDs.

AUTS2 has also been implicated in the aetiology of other neuropsychiatric disorders. Association studies using 65 nuclear multiplex autism families suggest that *AUTS2* may not be a candidate gene (Sultana *et al.*, 2002). Although, rare variants of this gene has been identified in cases of autism (Richler *et al.*, 2006) and ADHD (Elia *et al.*, 2010; Girirajan *et al.*, 2011). Interestingly, this gene has also been associated with schizoaffective disorder (Hamshere *et al.*, 2009) and epilepsy (Komoike *et al.*, 2010; Mefford *et al.*, 2010). Recently, our group has also found an association of *AUTS2* with alcohol consumption (Schumann *et al.*, 2011) which has also been replicated in another study (Zuo *et al.*, 2012). Additionally, expression analysis in post mortem hippocampal samples from patients with Alzheimer's disease (AD) showed that the *AUTS2* gene was upregulated compared to controls (Dahmcke *et al.*, 2008). Interestingly, *AUTS2* was one of the genes found to be hypermethylated in post mortem frontal cortical samples from schizophrenic males compared to matched controls (Mill *et al.*, 2008). Intriguingly, *AUTS2* is one of several genes originated from such molecular genetic studies that is thought to be involved in neurodevelopmental processes (Walsh *et al.*, 2008; Raychaudhuri *et al.*, 2009; Elia *et al.*, 2010; Glessner *et al.*, 2010; Mefford *et al.*, 2010; Pinto *et al.*, 2010; Piton *et al.*, 2011), which could explain its involvement in the aetiology of a broad range of neuropsychiatric disorders. Aberrant developmental processes such as increased neurogenesis and decreased neural apoptosis can contribute to brain abnormalities in neuropsychiatric disorders. These developmental aberrations may be a result of gene mutations or environmental factors which together or independently affect brain development leading to pathological phenotypes (Talkowski *et al.*, 2012). An example of one such gene is *DISC1*, a susceptibility gene for a wide range of mental disorders. It is expressed in neuronal progenitor cells and post-mitotic neurons in the developing cerebral cortex and is thought to play a key role in the transition from progenitor proliferation to post-mitotic neuron migration (Ishizuka *et al.*, 2011). Studies in mice show that overexpression of G-protein α subunit, Gαs, either during development or in adulthood, result in enlarged lateral ventricles and smaller dorsal and ventral striatum (Kelly *et al.*, 2009). Intriguingly, $GABA_A$ receptors in particular are reported to play a crucial role in the maturation of cortical circuits and synaptogenesis (Cancedda *et al.*, 2007). While in adulthood, GABA receptors are responsible for the majority of inhibitory transmissions, in embryonic and neonatal brains, these are excitatory. Deficiency in $GABA_A$

receptor β3 subunit (GABRB3), which is highly expressed in the developing brain, is thought to contribute to neurodevelopmental disorders like epilepsy and autism (Tanaka *et al.*, 2010). In fact, GABRB3 knockout mice display neurological deficits and are proposed to be models for autism (Pizzarelli & Cherubini, 2011). Evidently, the abnormal alteration in essential cellular processes such as cell proliferation, differentiation, migration and apoptosis may lead to pathological consequences on brain development and therefore function.

Genetic and neuroimaging studies are beginning to broaden our understanding on genetic differences on brain function between normal and disease populations. Perhaps a striking finding, which is now well established, is that whole brain, gray matter and white matter volumes are highly heritable (Peper *et al.*, 2007) with 90%, 82% and 88% heritability estimates respectively (Baare *et al.*, 2001). Further expansion on this finding comes from a report revealing a strong relationship between genes, anatomical brain structure and behaviour (Thompson *et al.*, 2001). Many studies have shown that aetiology of several psychiatric disorders may arise due to aberrant processes in brain development resulting in anomalous brain structure (Lainhart & Lange, 2011). Several independent studies have reported increased total brain volumes in individuals with autism as well as increased cerebellar hemisphere volumes (Brambilla *et al.*, 2003). A recent preliminary study showed that brain overgrowth in autistic males was due to an augmented number of neurons in the prefrontal cortex (Courchesne *et al.*, 2011) and is supported by another finding that reported increased neuron number and larger brain volume in individuals with autism (Lainhart & Lange, 2011). On the contrary, a number of MRI studies have shown that patients with schizophrenia possess reduced brain volume (Andreasen *et al.*, 1994; Gur *et al.*, 1994) reduced grey matter volume (Shelton *et al.*, 1988; Woods & Yurgeluntodd, 1991; Zipursky *et al.*, 1992),reduced cortical volume (Glahn *et al.*, 2008; Arnone *et al.*, 2009; Ellison-Wright & Bullmore, 2010) which may be a direct result of cortical thinning (Rimol, 2012) due to reduced synaptic density (Huttenlocher, 1979; Huttenlocher & Dabholkar, 1997). This may, to some extent, be due to decreased neural stem cell proliferation during adult neurogenesis (Reif *et al.*, 2006). Indeed, knockdown of *DISC1*, in adult and embryonic mouse stem cells revealed reduced neural progenitor proliferation and subsequent premature differentiation (Mao *et al.*, 2009). Interestingly, there is reportedly substantial overlap in the underlying brain morphological abnormalities in cerebral cortex and subcortical structures of schizophrenia and bipolar disorder patients. Both groups showed substantial sub cortical volume reductions in the hippocampus and the brain stem as well as ventricular enlargements (Rimol *et al.*, 2010; Rimol,

2012). Other neuropsychiatric disorders, although, not extensively researched report brain anatomy differences. White matter abnormalities in depression patients have also been evident from post-mortem and genetic studies (Tham *et al.*, 2011). Abnormal brain structure specifically enlarged medial temporal lobe and basal ganglia has been implicated in drug addiction (Ersche *et al.*, 2012) although it can be argued that this may be as a result of neuroadaptations occurring due to drug intake. A commonality emerging from the above findings is that defects in brain development contribute to the aetiology of a broad range of neuropsychiatric disorders. Abnormalities in brain development could be a result of genetic dysfunction. Thus there is an increasing need to study the function of neurodevelopmental genes such as *AUTS2*.

Of course as mentioned earlier, the aetiology of psychiatric disorders stems from the aberrant alteration of several genes rather than just one. It is important to consider epigenetic mechanisms such as DNA methylation and chromatin remodelling which change the gene expression without altering the DNA sequence of a gene. Indeed, vital neurodevelopmental processes, such as cell differentiation and neurogenesis, are highly regulated at the level of chromatin remodelling, histone acetylation and methylation. Dysfunction of MeCP2, a transcriptional repressor that binds to methylated DNA, is associated with several neurodevelopmental disorders as it is thought to cause a premature arrest in neuronal development (Gonzales & LaSalle, 2010). Another example, histone deacetylase (HDAC) inhibitors induce neural differentiation in embryonic cortical cells. Additionally, histone deacetylation is crucial for the timing of oligodendrocyte differentiation and myelination: administration of valproate (a non-selective HDAC inhibitor) leads to hypomyelination, delayed expression of differentiation markers and prolonged expression of progenitor markers in these cells. Abnormal DNA methylation as a result of deficiency in the DNA methyltransferase DNMT1, or the methyl-CpG binding protein MBD1, causes abnormal neuronal function and postnatal death or decreased neurogenesis respectively. It may be that AUTS2 gene dysregulation, in addition to other genes, could play a role in mediating abnormal brain development and function consequently associating it with various neuropsychiatric disorders. Obviously, this argument is speculative and as very little is known about *AUTS2* and its molecular mechanisms, functional studies are required to research into its role.

1.5 Functional Genetic Approaches – Gene Manipulation

Although genetic studies have led to the identification of susceptibility genes, functional genetic studies have provided convincing evidence to support the involvement of specific genes in disease development as well as contributed to elucidating their mechanisms of action. This will not only uncover the genetic architecture of disease pathology but may also lead to a better identification of therapeutic targets for treatment of psychiatric conditions.

1.5.1 Gene silencing

1.5.1.1 RNA interference (RNAi)

RNA interference (RNAi) was coined after the landmark discovery that injection of double-stranded RNA (dsRNA) molecules into the nematode *Caenorhabditis elegans (C.elegans)*, whereby one strand is complementary to the coding region of the target gene, resulted in specific gene silencing (Fire *et al.*, 1998; Montgomery *et al.*, 1998). It is an evolutionary conserved molecular mechanism involved in several biological processes in all species (Waterhouse *et al.*, 1998; Wianny & Zernicka-Goetz, 2000; Caplen *et al.*, 2001; Yang *et al.*, 2001). In recent years, the discovery of RNAi has led to its wide use as a tool for studying gene functionality (Hommel *et al.*, 2003). The RNAi pathway is mediated by double stranded non-coding RNA molecules of around 21 nucleotides called small regulatory RNAs, which silence a specific target gene of interest and with high potency (Hannon & Rossi, 2004).

RNAi mechanism

In humans, there are two main classes of small regulatory RNAs: short interfering RNAs which mediate RNAi by downregulating gene expression through cleavage resulting in RNA degradation and microRNAs which results in gene silencing by transcriptional repression (Figure 3). A comprehensive description of both classes of RNAi-mediating molecules is given below:

(1) Short interfering RNAs (siRNAs) are derived from long dsRNA molecules. The process of targeted gene silencing initiates with an RNase III enzyme complex Dicer, which cleaves the dsRNA at 21-25 nucleotide intervals generating a siRNA molecule that is characterized by 5′ phosphorylated end, a 19- nucleotide duplexed region and 2 nucleotide overhang and unphosphorylated 3′ end. This distinct dsRNA molecule is recognized by the

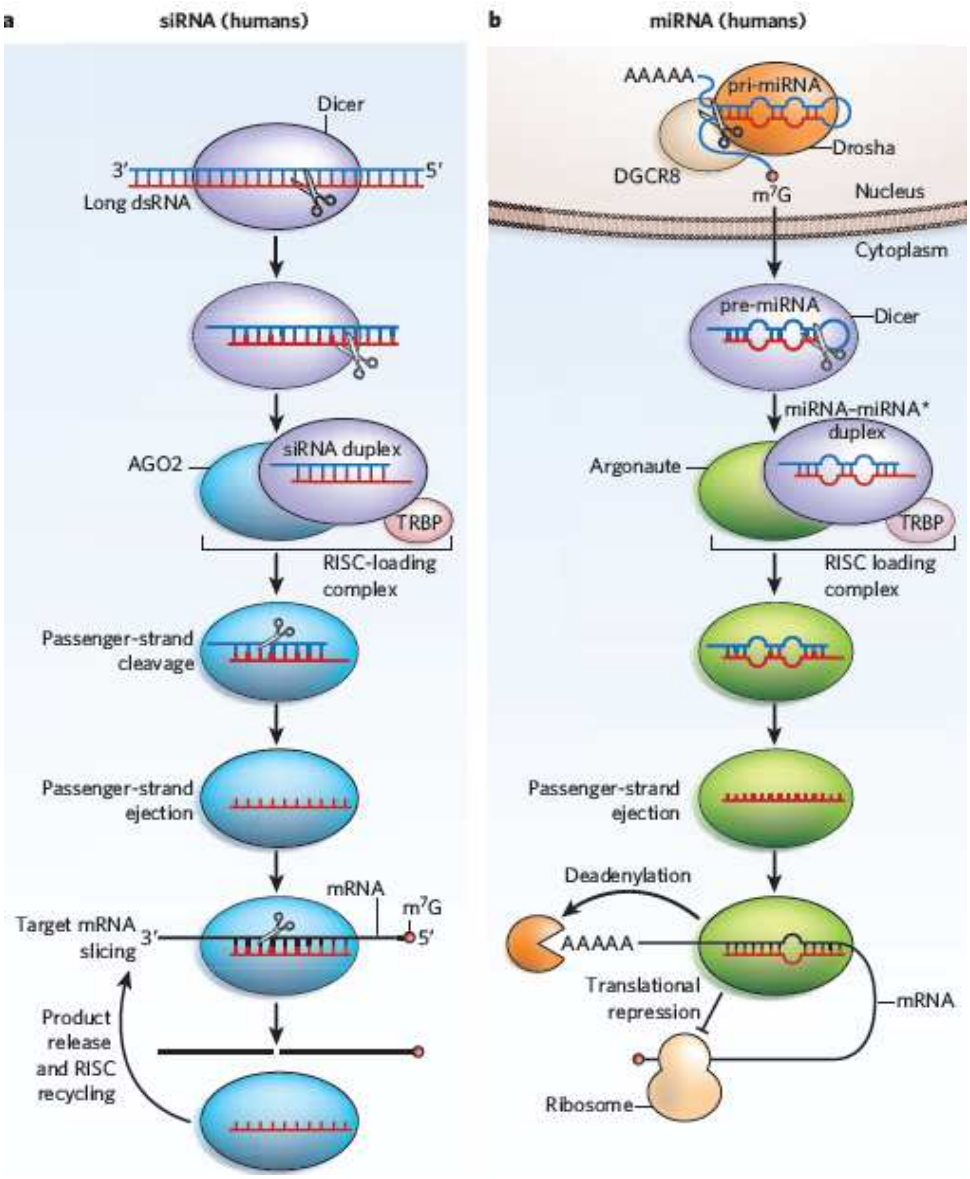
multiprotein complex RISC (RNA-Induced Silencing Complex), which unwinds the siRNA duplex and incorporates the single stranded antisense strand. The antisense strand guides RISC to the target mRNA, driving its endonucleolytic cleavage. The resultant antisense strand binds to the mRNA with sequence homology where nuclease Argonaute 2 (Ago2) cuts the mRNA leading to its degradation and resulting in targeted gene silencing (Hannon & Rossi, 2004; Meister & Tuschl, 2004).

Short hairpin RNA (shRNA) is another example of a long dsRNA and has long been used to confer sustained gene silencing for functional studies (Carmell *et al.*, 2003; Seibler *et al.*, 2005; Rubinson *et al.*, 2007). shRNA sequence comprises of the siRNA sequence (sense) followed by a loop structure and a reverse complement of the siRNA sequence (antisense). These are exported from the nucleus and are recognised and cleaved by the endonuclease Dicer, to form double stranded RNA similar to siRNA. For *in vitro* and *in vivo* experiments, the shRNA can be produced by the transcription of the shRNA plasmid construct mediated by Pol II or more commonly Pol III promoters (Paddison *et al.*, 2002; Singer & Verma, 2008).

(2) MicroRNA (miRNA) is encoded in the host genome and is transcribed as long primary transcripts called pri-miRNA from Pol II promotors (Kim & Kim, 2007). The pri-miRNA contains a hairpin structure that is cleaved by the nuclear enzyme Drosha and another dsRNA binding protein complex called diGeorge Critical region 8 (DGRC8) generating a ≈70nt miRNA precursor called pre-miRNA which is then transported to the cytoplasm and processed by Dicer, which generates the primiRNA to 19-25 nucleotide miRNA. These mature miRNAs are then incorporated in the miRNA-protein complex (miRNP) which includes RISC to induce silencing. Unlike siRNAs, the miRNAs pair with partial sequence complementarity to the target mRNA and lead to translational repression or decrease mRNA stability (Couto & High, 2010).

Figure 3 RNA interference (RNAi) mechanism taken from (Jinek & Doudna, 2009).

RNAi is mediated either via short interfering RNA (siRNA) or microRNA (miRNA). siRNA- mediated gene silencing involves degradation of mRNA whose sequence is complementary to the target sequence. On the other hand, miRNA anneal with partial sequence complementarity to the target mRNA and represses its translation.



Application of RNAi

Given that RNAi-mediated gene knockdown can achieve high knockdown efficiencies in mammalian cells, this technology holds great potential for gene function analysis *in vitro* and *in vivo* (Mittal, 2004). Effective gene silencing using RNAi has been demonstrated in neuronal cell lines (Gan *et al.*, 2002), neural stem cells (Wood *et al.*, 2003), primary mammalian neurons (Krichevsky & Kosik, 2002) and astrocytes (Frigeri *et al.*, 2002; Nicchia *et al.*, 2003). *In vitro*, shRNAs and miRNAs are widely used to induce loss of gene function to address neurobiological questions (Elbashir *et al.*, 2001; Harborth *et al.*, 2001; Tiscornia *et al.*, 2003; Dorsett & Tuschl, 2004; Parsi *et al.*, 2012). For example, *in vitro* RNAi-mediated gene silencing pointed out to the involvement of specific genes in embryonic developmental processes such as cell differentiation (Desclaux *et al.*, 2009), cell survival (Dickey *et al.*, 2011) and proliferation (Foskolou *et al.*, 2012), as well as the development of neural networks (MacLaren *et al.*, 2011)**.** These will be covered in more details in a later section.

Sustained gene silencing is achieved from viral vector plasmids expressing shRNA which is processed into siRNA in the cytoplasm. As discussed later, the silencing effects are sustained due to the recombinant viral DNA being permanently integrated into the host cell genome (Manjunath *et al.*, 2009). Interestingly, although the introduction of long double stranded RNA will elicit an interferon immune response in mammals, siRNA can circumvent this response (Manjunath *et al.*, 2009) making it applicable for *in vivo* studies. RNAi studies in various animal model such as *Drosophila* and *C.elegans* have contributed to our understanding at the cellular level of neural plasticity (Billuart *et al.*, 2001), axon guidance (Schindelholz *et al.*, 2001; Georgiou & Tear, 2002), synaptic endocytosis (Harris *et al.*, 2001) and neurodegeneration (Syntichaki *et al.*, 2002). At the behavioural level, rodents in particular, have been instrumental in unravelling the genetic underpinnings of many behavioural traits relating to addiction-like phenotype (Lasek *et al.*, 2007; Rewal *et al.*, 2009; Johnson & Kenny, 2010; Lasek & Azouaou, 2010), Parkinson's disease (Gorbatyuk *et al.*, 2010), Alzheimer's disease (Singer *et al.*, 2005) and anxiety-like behaviour (Eren-Kocak *et al.*, 2011). Most of these studies use viral vector for gene transfer discussed further in 1.6.2.

1.5.2 Generation of genetically altered mice

Mutant mice have proved invaluable to our understanding of molecular mechanisms that govern various behavioural and psychiatric traits. These studies have complemented and expanded on pharmacological approaches by enabling to investigate the role of specific genes (Grant *et al.*, 1992; Silva *et al.*, 1992; Phillips *et al.*, 1998; Wood & Toth, 2001; Blednov *et al.*, 2010). This approach has also considerably contributed to delineating the role of complex receptors for neurotransmitters through generation of mice with loss of function of individual isoforms to study behavioural phenotypes (Crabbe *et al.*, 1996; Phillips *et al.*, 1998; Ralph *et al.*, 1999; Scearce-Levie *et al.*, 1999; Loh & Ball, 2000; Wood & Toth, 2001; Jacobson *et al.*, 2007). The fact that the gestation times of mice are relatively short and the complete sequencing of the mouse genome also offer benefits to further understanding the function of any given gene. Briefly, to generate knockout or knockin mice, initially, the transgene, either a cDNA encoding a particular protein or an antisense fragment designed to silence the level of endogenous mRNA, is cloned downstream of a suitable promoter element to drive its expression. It is introduced into a pronucleus of a fertilized egg. Once inside the pronucleus, the transgene either integrates into the genome in a random fashion or is degraded by exonuclease activity. As the transgene insertion occurs early in the developmental process, most of the cells, if not all, will contain the transgene. However, if the transgene is integrated later in development, this will generate a mosaic transgenic.

Alternatively, transgenic mice may be obtained by a second route, using embryonic stem (ES) cells. These cells, which are derived directly from the culture of blastocysts, retain the ability to continue with embryonic development once injected back into an early mouse embryo. Introduction of exogenous DNA into the cultured ES cells allows one to generate transgenic mice via the production of germ-line chimeras. The gene of interest is modified in the ES cells by homologous recombination using a targeting vector that consists of a modified version of the endogenous gene. These targeting vectors also contain a gene conferring drug resistance (e.g. neomycin phosphotransferase, *neo*) for selection of recombinant ES cells. The advantage of this method, in contrast to the transgene pronuclear injection, is that desirable and low-frequency genetic alterations may be selected and verified in the ES clones maintained in culture and then reintroduced in the animal.

The downside of 'whole body' gene knockout/knockin is that all tissues express the transgene so it is difficult to separate the phenotype resulting from overexpression/ silencing a specific tissue, say the brain and other tissues. Thus methods such as conditional/ inducible

technologies are now available that allow *in vivo* gene manipulation at specific time periods and tissue specifically during or after development. The Cre/loxP recombination system is a widely used approach in which Cre is a recombinase that recognises and mediates recombination between loxP sites, excising the sequences in between. Two requirements need to be fulfilled for conditional targeting with the Cre–loxP system: (a) a 'floxed' allele must be created in such a way that two loxP sites flank an essential region of the gene, without altering its normal activity; (b) targeting of Cre expression must be tightly controlled: to that end, either transgenic mice by pronuclear microinjection or, better, knockin mice may be used.

This scenario allows spatial control of the occurrence of a mutation and therefore insight into the function of a gene in a particular cell type or tissue. There are a few limitations to this approach; it is an irreversible one-off event as well as it can be expensive and time-consuming to generate.

In vivo, a number of inducible systems have been developed to allow temporal and spatial regulation of transgene expression including the tet regulatory system which is widely used in transgenic mice. Briefly, the process involves a dual-transgene system whereby a specific promoter controls the expression of tTA (tet controlled Transcriptional Activator) and a second transgene encodes for a gene of interest under the control of tTA-responsive TetOp promoter. Thus, in the presence of tetracycline or its derivitive doxycycline, tTA is unable to bind to the TetOp promoter and expression is turned 'off'. A disadvantage of this system is that if the induction of the transgene is required only in adulthood, then the mice have to be persistently exposed to the tetracycline or doxycycline from conception, which can be very expensive and may also results in behavioural side effects. In fact, one study demonstrated that exposure to doxycycline resulted in impaired spatial memory and fear conditioning (Mayford *et al.*, 1996). Another disappointing feature of this approach is that transgene induction is limited by the rate at which the tetracycline or doxycycline is cleared from the body and can be variable between tissues (Kistner *et al.*, 1996; Chen *et al.*, 1998). Due to their limitations, gene delivery approaches are used to corroborate or extend upon findings obtained from transgenic animal models.

1.6 Gene delivery methods

1.6.1 Non viral method - Transfection

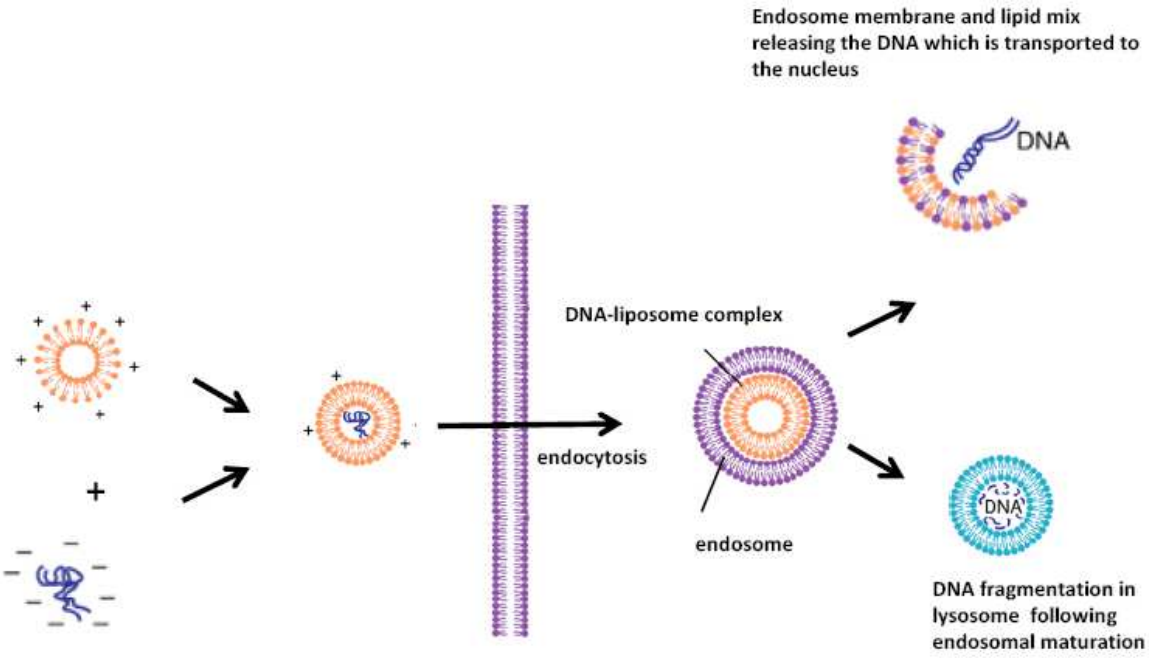
Transfection can be attained by introduction of nucleic acids into cells, which is essential for the study of gene and protein function. Different transfection methods are available for gene transfer although efficiencies differ especially in neural-derived cell lines which are often difficult to transfect.

One of the best established and commonly used methods of transfection is calcium phosphate DNA co-precipitation *in vitro*. Basically, it entails the formation of DNA crystals with calcium ions which precipitate onto the cells and is subsequently taken up by endocytosis. In proliferating cells the DNA can enter the nucleus when the nuclear envelope breaks down during mitosis. However, in postmitotic cells such as neurons, entry into the nucleus is more difficult and transfection efficiencies are low although attempts have been made to improve this (Goetze *et al.*, 2004). An advantage is that when optimised, calcium phosphate/ DNA co-precipitation results in good cell viability compared to other transfection methods (Karra & Dahm, 2010) such as electroporation.

Another transfection method that is widely used *in vitro* is lipofection which involves DNA and other genetic elements being complexed with a 'carrier' called liposomes that facilitate its uptake by the cells Figure 4. These are cationic lipids that, like cell membranes, possess long hydrophobic chains and a positively charged head group. DNA-liposome complex (lipoplex) formation results due to a negatively charged DNA molecule being encapsulated by a lipid bilayer not only protecting it from enzymatic degradation but also permitting its efficient uptake by endocytosis (Gao *et al.*, 2011). Indeed these carriers are among the most efficient non-viral vectors and have been used extensively to transfect various cell lines owing to the ability to transfect large nucleic acids and their ease of use. Yet again, in the case of neuronal transfection efficiencies remain to be low (Dalby *et al.*, 2004). Very few studies have used non-viral methods for gene delivery *in vivo*. Another important consideration is the duration of transgene expression as even the most efficient carrier such as PEI-mediated transfection in neuronal transfection results in expression in neurons that only lasts several days to few weeks (Abdallah *et al.*, 1996; Wang *et al.*, 2001). While some viral vectors have been demonstrated to have stable transgene expression in neurons that lasts for up to 6 months (Finiels *et al.*, 1995; Yamamura *et al.*, 2000); other advantages are explained in the next section.

Figure 4: Lipoplex-mediated transfection and endocytosis

Figure adapted from Parker et al. (2003). Negatively charged DNA and positively charged liposomes are combined to give a lipoplex, which enters the cell through endocytosis. The resulting endosome is either taken up by liposomes where the DNA is degraded or the DNA is transported in the nucleus for transcription.



1.6.2 Lentiviruses as vectors for gene delivery

To circumvent the abovementioned challenges and limitations, viral mediated gene delivery offer a highly efficient and sustained transgene expression in a variety of cell types, and is now increasingly being used to study gene function. Accordingly, viruses are used as gene vehicles that can be engineered for delivery of the desired sequences into host cells making it a useful tool for research and therapy. A range of viruses are available for transduction of genetic material *in vitro* and *in vivo* including herpes simplex virus derived vectors, adenoviruses, retroviruses (γ-retroviruses and lentiviruses), poxviruses and adeno-associated viruses. They have advantages and disadvantages as described in Table 1 below. For example, adenoviral vectors have very high transduction efficiency and can infect both dividing and non-dividing cells. They are also able to deliver large DNA particles of up to 38kb, however transgene

expression is shortterm as their DNA does not integrate into the host genome and they are highly immunogenic (Teramato *et al.*, 2000). While adeno-associated viruses are similar to adenoviruses but have lower immunogenicity and can integrate specifically into a site on chromosome 19 with no noticeable adverse effects but they have limited transgene capacity (up to 4.8kb) and require complicated process of vector production (Nayerossadat *et al.*, 2012).

Table 1: Comparision of viral vectors for gene delivery. *dsDNA = double stranded DNA; ssDNA = single stranded DNA.*

Viral vector	**Transgene capacity**	**Advantages**	**Disadvantages**
Adeno-associated vectors (ssDNA)	4kb	- Broad cell trophism - Infect dividing and non-dividing cells -Prolonged and high transgene expression -Low immunogenicity and non-pathogenic	-Difficult to achieve high titres. Require helper virus for replication.
Lentivirus (ssDNA)	10kb	-Broad cell tropism -Infect dividing and non-dividing cells -Stable and high transgene expression	-Possible insertional mutagenesis due to random integration into host genome. This could lead to oncogenicity.
Hepes simplex virus (dsDNA)	50kb	-Infect dividing and non-dividing cells -High titres achieved -Natural tropism to neuronal cells	-Possible toxicity -Risk of recombination -Transient transgene expression although prolonged in neurons
Adenovirus (dsDNA)	7-8kb	- Broad cell trophism - Infect dividing and non-dividing cells -High transgene expression -High titres achieved	-Capsid proteins trigger immune response to viral proteins. -Transient transgene expression

As lentiviral-mediated gene delivery has been used as a method of gene delivery in this thesis, this will be the focus of the following sub-sections.

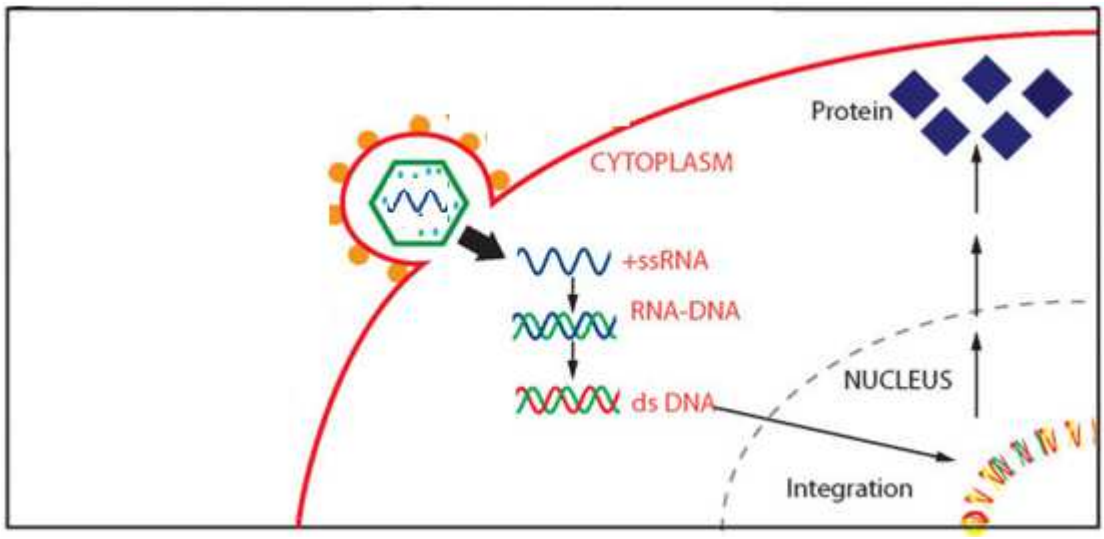
1.6.2.1 The lentiviral life cycle

Lentiviruses are a subset of retroviruses which are naturally occurring single stranded RNA-containing viruses. The life cycle of a retrovirus is typically divided into two stages as illustrated in Figure 5, infection and replication. The first phase involves the introduction of the viral genome into the cell, while replication entails the expression of structural and regulatory viral proteins that replicate the viral DNA and produce packaging proteins in order to produce lentiviral particles.

In detail, the lentiviral life cycle involves entry into the host cells through a fusion step via specific cell surface protein receptors encoded by the viral *env* gene. Upon entry into the cell cytoplasm, the viral RNA is reverse-transcribed into double stranded DNA by the viral enzyme reverse transcriptase, encoded by the *pol* gene. The viral DNA is then translocated into the cell nucleus where it is integrated permanently and randomly into the host cell genome (Cepko, 1988) due to the presence of long terminal repeats (LTRs). These are sequences that contain several repeats which flank functional genes and, in virus, are involved in the process of integration of viral DNA into cellular host DNA. As a result, all cells descended from an infected cell carry the viral genome. During cellular transcription and translation, the viral genomic RNA and viral structural proteins such as *gag* and *env* encoding for viral capsid and envelope proteins respectively, are produced and assembled to generate viral progeny. The unique features of lentiviruses are exploited for research purposes as described in the following section.

Figure 5: The lentiviral life cycle (Dottori *et al.*, 2011).

Receptors on the lentivirus capsid can fuse with the host cell membrane receptors allowing the introduction of the viral genetic material into the cell cytoplasm. Here the viral single standed RNA is reverse transcribed to double stranded DNA by RNA polymerase. The viral genome is then integrated into the host cell genome where with the help of host cell transcription and translation machinery will produce more RNA and viral packaging protein for production of viral progeny.



1.6.2.2 Modification of lentiviral features for use in research

A number of modifications are performed to convert natural viruses into useful tools for genetic modification for research and for biosafety. Alterations include the removal of viral genes responsible for virulence and pathogenesis, for instance, *vif* (induces G2 cell cycle arrest), *vpr* (alters cellular activation pathway), *vpu* and *nef* (both involved in the down regulation of the immune response) (Delenda, 2004). These 'accessory genes' are not essential for viral replication and are therefore absent.

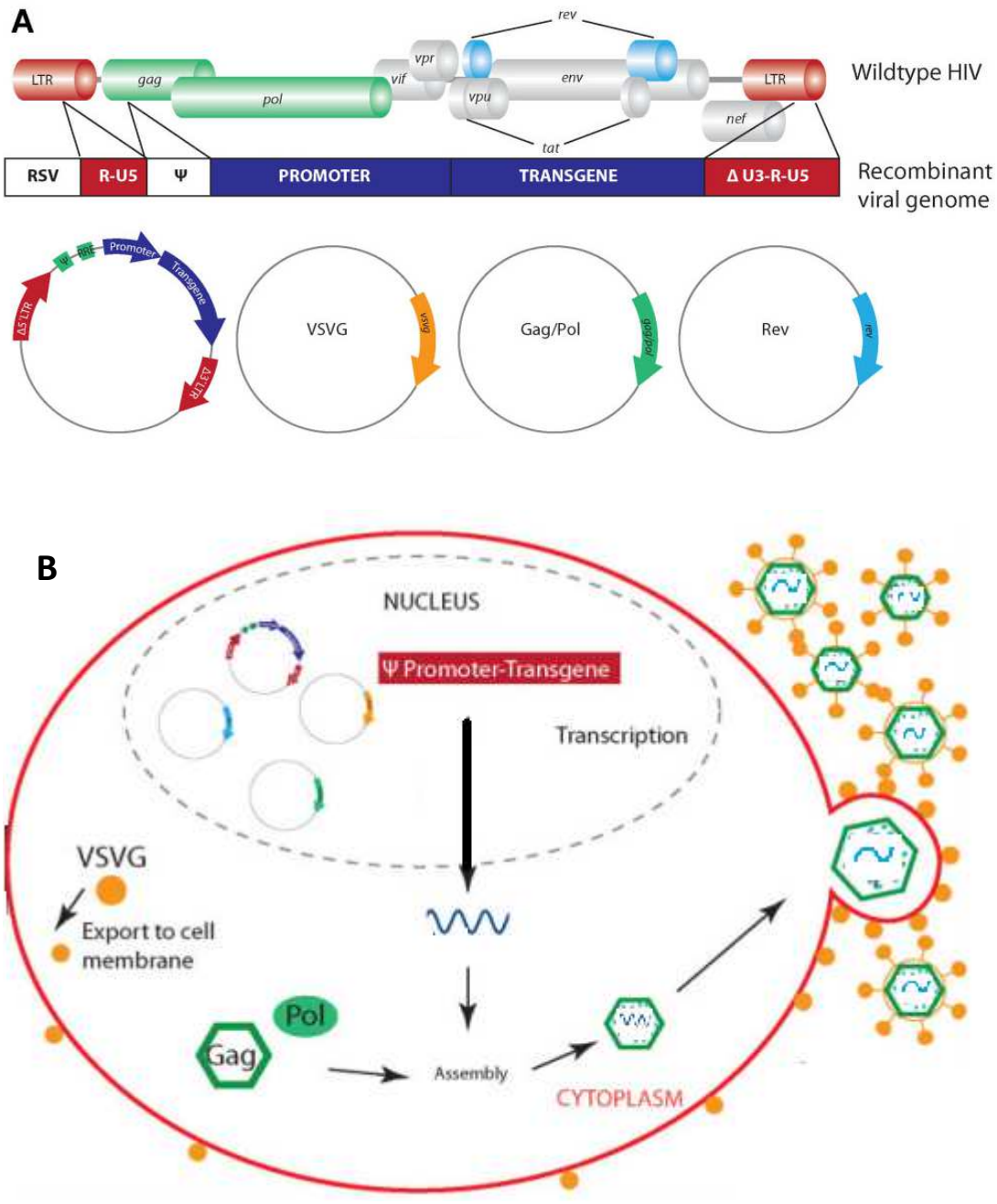
The entire lentiviral vector system is usually separated in three or four plasmids in the interest of improving bio safety: the packaging plasmid (may be split into 2 plasmids), the gene transfer plasmid and an envelope (*env*) plasmid. Hence, viral packaging can be achieved by using triple (2^{nd} generation) or quadruple (3^{rd} generation) co-transfection (Lever *et al.*, 2004) to generate lentiviruses that express the desired foreign genetic element (Figure 6B). Further modifications include the removal of the *env* gene from the lentiviral genome and replaced with a foreign glycoprotein vesicular stomatitis virus glycoprotein (*VSVG*) (Figure 6A). The presence of this protein confers the virus particle with the ability to transduce a wide range of cell types

including primary cells, stem cells and early embryos (Naldini *et al.*, 1996b; Lois *et al.*, 2002; Pfeifer *et al.*, 2002).

The lentiviral gene transfer expression plasmid vector also contains some alterations of the viral genome such as the incorporation of a transcriptional cassette with a promoter or a post transcriptional regulatory element that enhances transgene expression, like the human hepatitis virus post transcriptional element (HPRE), or the heterologous polyadenylation enhancer elements, as those derived from simian virus 40 (SV40) (Durand & Cimarelli, 2011). Transfer plasmid vectors also contain altered LTRs to facilitate the integration of the transgene into the host cell genome Figure 6A. LTRs are essentially modified by removal of viral enhancers and other transcriptionally active sequences from the 3'LTR resulting in a self inactivating (SIN) LTR (Yu *et al.*, 1986) which is thought to be safer than using native viral LTR. In addition, the lentiviral gene transfer plasmid vector contains several features that make it useful for its application *in vitro* and *in vivo*. For instance, it includes a strong promoter for gene expression and a fluorescent reporter gene, for example GFP (green fluorescent protein) or RFP (red fluorescent protein). As mentioned earlier, the viral gene transfer vector has been modified to contain a multiple cloning site for incorporation of foreign genetic materials e.g. a gene of interest or a short interfering RNA (siRNA). For gene overexpression, the open reading frame of the cDNA is amplified and cloned into the lentiviral expression gene transfer plasmid vector. In order to obtain sustained knockdown of a target gene, siRNA such as short hairpin RNA (shRNA) can be cloned into the gene transfer vector. On the whole, the resulting recombinant viral genome thus converts the infection process to an efficient transduction one, allowing for non-replicative entrance of a virus into the host cell followed by expression of the desired genetic information it carries in its genome. Accordingly, in the interest of biosafety, the resultant viruses produced are replication-incompetent, hence unable to produce viral progeny.

Figure 6: Genetic elements required for third generation lentiviral vector co-transfection system for lentiviral production taken from (Dottori *et al.*, 2011).

A) *This system includes 4 plasmids for co-transfection: Recombinant gene transfer plasmid in which some genes from the wild type lentiviral genome have been deleted or modified; genes essential for viral production are encoded on different plasmids. VSVG (env) plasmid conferring a broad range of cell type tropism for infection; the Gag/pol encodes the capsid proteins and reverse transcriptase and Rev which contains the nuclear localisation sequence necessary for gene transportation into the nucleus. B) Lentiviral production using third generation lentiviral packaging system upon co-transfection of packaging plasmids and transfer vector.*



1.6.2.3 Advantages and applications of lentiviral mediated gene delivery

The most widely used application of lentiviruses is to transduce desired genetic elements for investigating gene function. Owing to the envelope modification, lentiviruses are beneficial as they are able to transduce a wide range of cell types *in vivo*, including neurons, astrocytes, oligodendrocytes, primary hippocampal neurons and adult neural stem cells depending on the type of promoter used (Janas *et al.*, 2006; Hioki *et al.*, 2007). Indeed, lentiviruses can infect both dividing and non-dividing cells, and is particularly useful in carrying out gene modification in neurons, which are generally difficult to transfect (Lo *et al.*, 2001; Washbourne & McAllister, 2002). Lentiviruses also offer an added advantage in that they have a moderately large insert capacity for transgenes (up to 10kb) (Cazzin & Ring, 2010) for investigating gene function by overexpression in a cell line or *in vivo*. Lentiviral mediated gene silencing has also been used to identify genes important in development (Gibson & Ma, 2011). Several reports have demonstrated that efficient transduction in mouse and monkey embryonic stem cells (Hamaguchi *et al.*, 2000; Asano *et al.*, 2002; Pfeifer *et al.*, 2002). Moreover, it has been reported that transgene expression was not silenced throughout undifferentiated proliferation, as well as after differentiation *in vivo* and *in vitro (Gropp & Reubinoff, 2006)*. Lentiviral-based vectors overexpressing specific transcription factors could direct the differentiation of hESCs toward a desired cell lineage or cell type including neuronal progenitors cells which could be useful for regenerative medicine.

Of note, a beneficial feature of lentiviruses, especially for *in vivo* functional studies, is that normal cellular functions are not disrupted and no significant immune response is induced (Kordower *et al.*, 2000b). Numerous studies have shown that lentiviral vector successfully target neuronal cells in the rodent brain (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997; Zufferey *et al.*, 1997). The ability of the lentiviral genome to integrate permanently and stably in the host genome resulting in sustained gene modulation (Naldini *et al.*, 1996a; Gascon *et al.*, 2008) enables the study of behavioural consequences *in vivo* over extended time periods (Georgievska *et al.*, 2004). Lentiviruses also offer localised delivery permitting study of gene manipulation in particular brain regions of interest associated with a given behavioural trait (Caceda *et al.*, 2005; Lasek *et al.*, 2007; Land *et al.*, 2009; Ahmed *et al.*, 2010; Johnson & Kenny, 2010; Lasek *et al.*, 2010; Wang *et al.*, 2010). Numerous studies have investigated the role of specific genes using lentiviral based genetic modification in animal models to investigate their contribution to the development of experimental models of neuropsychiatric conditions including anxiety-like disorders (Rubino *et al.*, 2008; Eren-Kocak *et al.*, 2011), addiction-like phenotypes (Boyer & Dreyer, 2007; Johnson & Kenny, 2010; Lasek *et al.*, 2010)

and Parkinson's Disease (Ahmed *et al.*, 2010). For example, the growth factor GDNF (glial-derived neurotrophic factor) has been shown to prevent neurodegeneration and promote regeneration in many animals models of Parkinson's disease (Deierborg *et al.*, 2008). Recombinant lentiviral vectors have been developed and used for GDNF gene delivery (Kordower *et al.*, 2000a; Brizard *et al.*, 2006) including *in vivo* gene delivery. Interestingly, intracerebral injection of lentiviral into lesioned rats and rhesus monkeys displayed improvements in locomotor function (Dowd *et al.*, 2005). Also, in the brain, intra-striatal lentiviral mediated gene delivery of GDNF demonstrated complete striatal dopamine innervation which resulted in improved motor function in a rat model of early Parkinson's disease (Brizard *et al.*, 2006). Despite its limitations, lentiviral vectors hold great promise for gene therapy for this disease and, lentiviral delivery of GDNF and other neurotrophins are currently under Phase II clinical trials (Coune *et al.*, 2012).

1.7 Thesis Aims

The overall aim of this study was to functionally characterise Dopamine D2 and D3 receptors (D2R and D3R respectively) using lentiviral mediated gene delivery technique as well as investigate the possible function of a gene with as yet unknown function, Autism susceptibility candidate 2 (*AUTS2*). Specifically the aim of this thesis is sub-divided into the following:

1) Establish lentiviral mediated gene delivery technique *in vitro* and *in vivo* by delineating the role of two well characterised genes D2R and D3R on locomotor behaviour.

2) Elucidate the cellular localisation of AUTS2 and identify its protein interacting partners.

3) Given its potential characterisation as a neurodevelopmental gene, investigate the consequence of manipulating *AUTS2* expression on global gene expression in a newly established human foetal neural stem cell line, SPC-04.

Chapter 2 Materials and Methods

This chapter details the generalised protocols used in the research reported in this thesis. Specialised methods, specific to certain experiments, are reported separately in the relevant chapter(s).

Note:

- *Solutions and buffers were prepared using ultrapure double distilled water (ddH_2O) from an Elga Maxima water purification system, unless otherwise stated.*
- *For tissue culture, all buffers and reagents including dH_2O were tissue culture grade.*
- *Molecular biology grade water and ethanol was obtained from Sigma, UK.*
- *Restriction enzymes used throughout the chapters were purchased from New England Biolabs (NEB), UK.*
- *Through this thesis, RT indicates room temperature.*

2.1 Tissue culture

2.1.1 Materials

2.1.1.1 Cell lines

Four different cell lines were used in this thesis:

i) **Neuro2a** – a clone established from a spontaneous neuroblastoma tumor of a strain of albino mouse (Klebe & Ruddle, 1969), was purchased from American Type Cell Collection (ATCC), USA.

ii) **HEK 293T** – a highly transfectable cell line derived from human embryonic kidney, (ATCC, USA). An SV40 Large T-antigen is present which enables episomal replication of transfected plasmids containing the SV40 origin of replication.

iii) **293FT** – is a variant of the HEK 293T cell line, from Life Technologies, UK.

iv) **SPC-04** – Human foetal neural stem cells, are conditionally immortalized cell line, generated from 8 week old human fetal spinal cord as described in (Kubinova *et al.*, 2010), a kind gift from Dr Graham Cocks and Prof. Jack Price (James Black Centre, King's College London). The SPC-04 cell line was immortalized using a retroviral vector encoding for c-myc-ERTAM, that is c-myc fused to a mutant of the estrogen receptor sensitive to 4-OHT. Expression of this fusion protein enables cell immortalisation hence resulting in stable and continuous propagation while maintaining phenotypic and karyotypic stability, in the presence of 4-OHT. In the absence of 4-OHT, c-myc is not active and the cells cease to propagate.

2.1.1.2 Media and supplements

Table 2 contains details of the media and supplements for each cell line. Dulbecco's Modified Eagles medium (DMEM) and Fetal Bovine Serum (FBS), 2mM L-Glutamine, 100U/ml Penicillin and 100µg/ml Streptomycin were purchased from Sigma, UK. All other reagents were purchased from Life Technologies, UK. Where FBS was required to be heat inactivated, this was done by incubation at 56°C for 45 minutes before addition to the media.

Table 2: List of cell lines and details of the media required for their culture

Cell line	Media and supplements
Neuro2a	DMEM containing 4.5g/L glucose and 2mM L-Glutamine supplemented with 10% heat inactivated FBS, 100U/ml Penicillin and 100µg/ml Streptomycin.
HEK 293T	
HEK 293FT	DMEM containing 10% FBS (non heat-inactivated) supplemented with 0.1mM MEM-non-essential amino acids, 1mM sodium pyruvate, 6mM L-glutamine and 500µg/ml Geneticin® .
SPC-04	Refer to Table3 and section 2.1.1.3 for details.

Table3: RMM+ media preparation for SPC-04 cell line.

Components for RMM+	Final concentration	Source
DMEM:F12 (Ham)	-	Life Technologies, UK
Albumin serum, Human (HAS)	0.03%	Baxter Healthcare Ltd., UK
Human Apo-Transferrin	100µg/ml	Sigma, UK
Insulin, Human recombinant	5µg/ml	Sigma, UK
L-Glutamine	2mM	Sigma, UK
Progesterone	60ng/ml	Sigma, UK
Putrescine Dihydrochloride, Human	16.2µg/ml	Sigma, UK
Sodium Selenite	40ng/ml	Sigma, UK
4-hydroxy-tamoxifen	100nM	Sigma, UK
Human EGF	20ng/ml	PeproTech EC Ltd, Germany
Human bFGF	10ng/ml	PeproTech EC Ltd, Germany

2.1.1.3 SPC-O4 media and reagents

SPC-04 cell lines were maintained in reduced modified media (RMM) with growth factors and 4-hydroxy-tamoxifen (4-OHT) denoted (RMM+). This media was filtered through a 0.2µM filter unit (Stericup; Millipore, UK) and was stored for no more than 4 weeks at 4°C. Suppliers for individual components and final concentrations required for RMM+ preparation are detailed in Table3: RMM+ media preparation for SPC-04 cell line. Stock solution preparations for individual components are given in Table 4.

Table 4: Stock solutions prepared for some of the components in RMM+

Component	Stock solution preparation
Albumin serum, Human (HAS)	20% (w/v; no preparation required)
Apo-Transferrin, Human	50mg/ml diluted in DMEM:F12 containing 1mM HEPES pH 7.4
Human recombinant insulin	10mg/ml (w/v; no preparation required)
L-Glutamine	200mM (no reparation required)
Human Putrescine dihydrochloride	8.1mg/ml dissolved in tissue culture grade water
Sodium selenite	20µg/ml dissolved in DMEM:F12
Progesterone	1mg dissolved first in 100µl molecular biology grade ethanol and then diluted in DMEM: F12 to give 20µg/ml
4-OHT	1mM dissolved in molecular biology grade ethanol
EGF,Human	10µg/ml dissolved in DMEM:F12 containing 1mM HEPES and 0.03% human albumin serum
b-FGF, Human	10µg/ml dissolved in DMEM:F12 containing 1mM HEPES and 0.03% human albumin serum

2.1.1.4 RMM without growth factor and 4-OHT (RMM-)

RMM without growth factors and 4-OHT denoted RMM- was used to induce and maintain differentiation in the SPC-04 cell line. This media contained all components in Table3, except for EGF, b-FGF and 4-OHT.

2.1.1.5 Preparation of differentiation inducing agents

Two stock solutions were prepared in order to induce differentiation in SPC-04 cells: DAPT (LY-374973 N-[N-(3,5-**D**ifluorophenacetyl)-L-**a**lanyl]-S-**p**henylglycine **t**-butyl ester) and ATRA (**A**ll-**t**rans **r**etinoic **a**cid). Both stock solutions of 10mM DAPT and 10mM ATRA were prepared in DMSO, aliquoted and stored at -80°C.

2.1.1.6 Preparation of trypsin inhibitor solution (Trit)

Trit consisted of DMEM:F12 containing 1% Human Albumin Serum, 0.55% soyabean trypsin inhibitor (v/w) and 0.025U/ml bensonase solution (Merck, UK).

2.1.1.7 Growth surface for adherent cell lines

All flasks, plates and other growth surfaces for 293T, 293FT and Neuro2a cell lines were purchased from BD Biosciences, UK.

All flasks and 6-well plates used for SPC-04 cell culture or growth were Nunclon™ Δ Delta surface treated to ensure excellent conditions for cell attachment and growth purchased from Nunc. UK.

2.1.2 Methods

2.1.2.1 Maintenance of all cells lines

Cell lines were maintained at 37°C with 5% CO_2, humid incubator. All reagents were pre-warmed before use. Cells were propagated until reaching approximately 80% confluency and were passaged as detailed in 2.1.2.2 for SPC-04 cell line and 2.1.2.4 for other cell lines.

2.1.2.2 Passaging SPC-04

Appropriate size culture flasks were coated with a thin layer of 20µg/ml laminin (diluted in from a 1mg/ml stock in cold DMEM:F12) for three hours in a 37°C with 5% CO_2, humid incubator. Just prior to plating cells, the excess laminin was aspirated off and the flask rinsed with DMEM:F12 to remove residual laminin. This was aspirated and replaced with RMM+ and returned to the incubator. Next, the culture flask to be passaged was first rinsed with HBSS (Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+}; Life Technologies), followed by immediate addition of TrypZean (Lonza, UK) and returned to the incubator for approximately 2 minutes. Upon confirmation of cell detachment under an inverted microscope, Trit was added to inactive the enzyme and the cell suspension was centrifuged at 900rpm for 5 minutes to give a pellet which was resuspended in a small volume of RMM+ for either freezing and/or plated for further propagation in a laminin coated flask at approximately 20,000 cells/cm^2.

For freezing, a 20% DMSO solution (diluted in RMM+ was added to an equal amount of cell suspension to give a final concentration of 10% DMSO. These were, frozen at 1°C/min in a freezing container (Mr. Frosty, Nalgene®) with isopropanol and stored at -80°C overnight and transferred to liquid nitrogen.

2.1.2.3 Inducing differentiation of SPC-04

For inducing differentiation, cells were seeded at 12,000 cells/cm^2 in 6-well plates and allowed to grow over two days. Subsequently, cells were rinsed twice in RMM- to ensure removal of growth factors before RMM- containing a final concentration of 10µM DAPT and 100nM ATRA was added to the cells and incubated for 48 hours. This experimental stage is referred to as 'pre-differentiation' hereafter. Following this, the medium is changed to RMM- only, every two days until the end of the experiment. For reference to the experimental stage, differentiation begins after pre-differentiation thus '3 days differentiation' refers to 3 days after pre-differentiation stage. Although it is challenging to quantify such level of differentiation, the above experimental stages were used for referencing and comparision purposes for the experiment. Of course this does not reflect or compare to the 'level' of differentiation *in vivo*.

2.1.2.4 Passaging other adherent cell lines

293T, 293FT and Neuro2a cell lines were passaged at a 1:10 split ratio. This process involved removing the culture medium and rinsing the adherent cells with 1x Dulbecco's phosphate buffered saline (PBS) and incubating the adherent cells with a thin layer of 0.05% (1x) trypsin-EDTA solution (Life Technologies, UK) at 37^{o}C for 5 minutes; or until the cells detached from the flask. Complete medium was added to the detached cells and centrifuged at 1500rpm for 5 minutes at RT. The cell pellet were resuspended in fresh complete medium, diluted accordingly and plated. Cells were maintained at 37°C with 5% CO_2, humid incubator.

For long term storage, cells were harvested by trypsinisation and spun down at 1500rpm for 5 minutes. The resulting pellet was resuspended in freezing medium (10% dimethyl sulphoxide [DMSO], 90% appropriate culture medium) ensuring between 2 – 5 x10^{6} cells were frozen per ml and transferred to cryotubes. These were, frozen at 1°C/min in a freezing container (Mr. Frosty, Nalgene®) placed at -80^{o}C overnight and transferred to liquid nitrogen storage.

2.1.2.5 Cell counting

For determining cell count, the trypsinisation protocol appropriate for each cell line was carried out for obtaining cells in suspension, and counted using a NucleoCounter NC-100 (Chemometec, UK). Equal amounts of cell suspension, Reagent A (lysis buffer; Chemometec, UK) and Reagent B (stabilising buffer; Chemometec, UK) are mixed to give a minimum volume of 120µl. This mixture is then introduced into a Nucleocasette which contains an immobilized fluorescent dye propidium iodide (PI), in its interior. The PI intercalates with the DNA with 1 dye molecule per 4-5 DNA base pairs. The nucleocassette is placed into the NeucleoCounter, the cellular DNA – PI stained mixture is automatically transferred to the measurement chamber. Green light excites the PI-DNA mix and the resulting red light emitted is registered and registered in the CCD camera for correlation into a cell count. Concentration was given in cells/ml. The dilution factor was multiplied with this figure to give final concentration of the cells in suspension obtained following trypsinisation.

2.2 Transfection

2.2.1 Materials

Unless otherwise stated, all reagents used in this section were purchased from Sigma, UK. Tissue Culture Grade dH_2O was used to prepare reagents and buffer. These were filtered using a sterile 0.22µm pore filter (Sartorius Stedim).

2.2.1.1 Hepes Buffered Saline (HBS), 2x

281mM NaCl, 100mM HEPES, 1.5mM Na_2HPO_4, pH 7.12. Aliquoted and stored at -20°C.

2.2.1.2 $CaCl_2$, 2.5M

2.5M $CaCl_2$ was prepared in dH_2O.

2.2.2 Methods

2.2.2.1 Calcium phosphate transfection

The day before transfection, cells were seeded as per Table 5 below, depending on size of plate or dish used. On the day of transfection, the medium was replaced with fresh culture medium and placed back in the incubator. Plasmid DNA and 2.5M $CaCl_2$ as described in Table 5 were added together and mixed. Then 2x Hepes Buffered Saline (HBS) pH 7.12 was slowly added in a dropwise manner whilst applying air pressure to generate a bubbling effect to mix the DNA/$CaCl_2$/HBS solutions. This mix was then left at RT for 10 minutes to allow for the calcium DNA precipitates to form. Next, all of this mix was added to the medium and placed back in a humidified incubator at 37°C with 5% CO_2. The following day, the medium was replaced with fresh culture medium. Cells were observed microscopically 48 hours after transfection to detect GFP expression. 72 hours after transfection, cells were either lysed for RNA and/or protein extraction (detailed in section 2.7 and 2.9 respectively) or trypsinized into 8 chamber slides for immunostaining (see sections 2.1.2.4 and 2.1.2.5).

Table 5: Details of reagents and cell for $CaCl_2$ – mediated transfection.

	10cm dish	**6 well**
Number of cells plated	$3x10^6$ in 10ml culture medium	$5x10^5$ in 3ml culture medium
Culture medium volume on the day of transfection	5ml	1ml
Plasmid DNA	20µg	3µg
Total volume with dH_2O	450µl	76.5µl
2.5M $CaCl_2$	50µl	8.5µg
2x HBS pH7.4	500µl	85µl
Volume added to cells	1ml	170µl

2.2.2.2 Determining transfection efficiency

Transfection efficiency was determined approximately by dividing the number of GFP+ve cells over the total number of cells in the field of view at 20x magnification (atleast 100 cells /field). Three or four random areas of the plate/ well/slide were chosen to corroborate the results.

2.3 Lentiviral production, transduction and concentration

2.3.1 Materials

Unless otherwise stated, reagents used in this section were purchased from Sigma, UK. All tissue culture buffers and reagents were filtered using a sterile 0.22μm pore filter. The lentiviral plasmids used for lentiviral production have been described in further details in the specific data chapters.

2.3.1.1 Polybrene, 1000x

8mg/ml polybrene was prepared in Tissue Culture Grade H_2O.

2.3.1.2 Chloroquine, 100mM

100mM chloroquine was prepared using Tissue Culture Grade H_2O.

2.3.2 Methods

2.3.2.1 Lentiviral production

Various transfection protocols were applied in this thesis and are described in detail in the relevant data chapters.

2.3.2.2 Lentiviral Transduction

This protocol has been previously optimised and used in the laboratory by Dr. Sylvane Desrivieres.

Lentiviral transduction was carried out to 1) determine viral titres, 2) investigate knockdown efficiency of putative shRNAs *in vitro* and 3) verify the transcriptional gene induction efficiency of overexpression constructs. The following protocol is specific to the lentiviral transduction of 293T cell line. The day before infection, cells were plated either at $1x10^5$ (for 24-well plates) and $5x10^5$ (for 6-well plates). On the day of infection, the lentiviral supernatants were added to the cells in a total volume of 1ml medium (for 24 well plates) and 3ml medium (for 6 well plates) with a final concentration of 0.4mg/ml Polybrene in order to facilitate the fusion of the virus particle to the cell membrane hence increasing the infection efficiency. The plates were centrifuged for 90 minutes at 2500rpm at RT and then placed for 2.5 hours at 37°C in a 5% $C0_2$

humid incubator after which the medium was replaced and incubated back for 72 hours. Prior to harvesting, cells were observed under a fluorescent microscope for the presence of GFP and estimation of transduction efficiency. The cells were lysed for RNA extractions (section 2.7.2.2 below) and/or protein extraction (section 2.9.1 below).

2.3.2.3 Concentration of lentivirus

This protocol has been established in our laboratory previously. To concentrate the lentiviral particles, the filtered supernatants were ultracentrifuged at 75000xg (Beckman Coulter, UK; SW27 Rotor) at 4°C for 1.5 hours. The supernatant was removed leaving a small visible pellet containing viral particles to which cold PBS was added and left overnight at 4°C. The pellets were resusupended the following day and stored in aliquots at -80°C.

2.3.2.4 Lentiviral quantification

Virus titers were determined by transducing the 293T cell line (see 2.3.2.2 above) and measuring eGFP expression by FACS analysis. To estimate the concentration of functional transducing units/ ml, 293T cells were transduced with serial dilutions of an aliquot of each vector preparation. Briefly, titration of concentrated virus was carried out in 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6}$and 10^{-7} dilutions and added to the cells with medium containing 1x polybrene (final concentration 0.4mg/ml). Cells were centrifuged for 90 minutes at 2500rpm at RT and then incubated for 2.5 hours at 37°C in a 5% $C0_2$ incubator after which the medium was replaced and incubated back. After 72 hours, cells were harvested and subjected to flow cytometric analysis.

The transducing units (TU/ml) were calculated as shown in the equation below:

TU/ml = (%GFP+ cells/100) x nb x dilution factor

A dilution giving ~10-15% GFP+ cells were chosen which represents one virus integration site per cell and nb = *number of cells plated x* $2^{\textit{number of days after plating}}$.

The standard titre obtained from using the transfection protocol in this thesis was 1 -3 x10^9 TU/ml. To ensure the same concentration of lentivirus was used for experiments, the lentiviral particles were diluted in 1x PBS to adjust for different titres.

2.3.2.5 Infection of SPC-04 with lentivirus

Transduction of the SPC-04 cell lines was carried out at the pre-differentiation stage in 6-well plates (9cm^2). A higher density of cells was plated to account for apoptosis occurring as a result of differentiation and lentiviral infection. Thus, 20x10^3 cells were plated 24 hours prior to adding RMM- medium containing 10µM DAPT and 100nM ATRA and a concentration of 0.26x10^6 TU/ml of lentivirus per well. The cells were incubated with this medium for 48 hours before replacing with RMM- medium.

2.4 Bacterial transformations and cultures

2.4.1 Materials

All reagents, solutions and chemically competent bacteria used in this section were purchased from Life Technologies, UK, unless otherwise stated.

2.4.1.1 Ampicillin stock solution

Ampicillin (Sigma,UK) was prepared at a stock solution of 100mg/ml in ddH_2O and was aliquoted and stored at -80°C.

2.4.1.2 Lennox L Broth base (LB)

Lennox L Broth base (LB) was made by dissolving 20g of this powder in a litre ddH_2O giving the following constituents per litre: 10g SELECT Peptone 140, 5g NaCl and 5g SELECT yeast extract, pH 7.0. The broth solution was autoclaved at 121°C for 15 minutes. Before use, ampicillin stock solution was added to the broth to give a final concentration of 100µg/ml and stored at 4°C.

2.4.1.3 LB Agar

LB-Agar was also purchased as a pre-mixed powder consisting of 1.2% (w/v) SELECT – agar in LB. A 3.2% (w/v) solution of LB-agar was prepared in ddH_2O and autoclaved 121°C for 15 minutes before use. Before cooling completely, ampicillin stock solution was added to give a final concentration of 100µg/ml. This was poured into 10cm diameter sterile petri dishes and allowed to solidify overnight. The plates were stored at 4°C for up to 8 weeks.

2.4.1.4 Bacterial strains

Transformations were carried out in chemically competent *Escherichia coli (E. coli)* host strains either TOPO® One Shot TOP10 or MAX Efficiency® Stbl2™ competent cells which are a derivative of the JM109 bacterial cells and are suitable for cloning unstable inserts such as retroviral sequences or direct repeats.

2.4.2 Methods

2.4.2.1 Transformation of E.coli with plasmid DNA

Plasmid DNA (1-50ng) were transformed into 50µl chemically competent cells and incubated on ice for 30 minutes. This was followed by heat-shock at 42°C for 45 seconds and returned to ice for 2 minutes. 900µl of S.O.C medium was added and the cells were allowed to recover at 30°C, shaking at 225rpm for 1.5 hours. The cells were then centrifuged for 5 minutes at 2500rpm and resuspended in 300µl LB media. 100µl of the cell suspension was plated on LB-agar plates containing ampicillin as described in 2.4.1 and incubated overnight at 30°C to isolate individual colonies.

For TOPO® One Shot TOP10 competent cells, the above protocol was carried out except following heat shock, cells were allowed to recover at 37°C, shaking at 225rpm for 1 hour. And, upon plating on LB agar plates, were incubated at 37°C overnight to isolate individual colonies.

Bacterial colonies growing on LB agar plates were individually isolated aseptically and grown as precultures by inoculating in 5ml of LB broth containing 100µg/ml ampicillin with gentle shaking at 225rpm overnight at 37°C for TOPO® One Shot TOP10 Chemically Competent E. Coli and at 30°C for MAX Efficiency® Stbl2™ Competent Cells.

2.5 Quantification of DNA and RNA

The concentration and purity of DNA were determined by UV spectrophotometry using a NanoDrop 1000 spectrophotometer (Fisher Scientific, UK). 1µl stock DNA was loaded onto the optical pedestal and the absorption at 260nm indicated the concentration of DNA in the sample after selecting the double-stranded DNA quantitation option. The same was done for RNA quantification, only the single-stranded RNA option was selected. The absorbance of samples were read at 260nm and 280nm (A_{260} and A_{280}). DNA can be quantified at 260nm; a solution of DNA with an optical density (OD_{260}) of 1.0 has a concentration of 50ng/µl (using a 1mm path length). A_{260} / A_{280} absorbance ratio gives an indication of the purity of the sample, with pure DNA having a value of 1.8. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb at or near 280nm.

2.6 DNA extraction and analysis

All DNA extraction kits used in this section were purchased from QIAGEN, UK unless otherwise stated. All buffers and other reagents used here were obtained from Sigma, UK unless otherwise stated.

2.6.1 Materials

2.6.1.1 DNA Agarose gel

DNA was separated by electrophoresis on a 0.8% - 2% (w/v) agarose gel made up in 1x TBE buffer, supplemented with 0.1µg/ml of ethidium bromide when sufficiently cooled.

2.6.1.2 Gel loading buffer

0.25% (w/v) bromophenol blue, 0.25% xylene cyanol FF; 30% (w/v) glycerol in water.

2.6.2 Methods

2.6.2.1 Mini- and Maxi- preps

For minipreps, 3ml of bacterial culture was centrifuged at 2500 rpm for 5 minutes. Minipreps were carried out using the NucleoSpin Plasmid DNA Purification Kit (Machery-Nigel GmbH & Co. KG, Germany) according to the manufacterer's instructions. Briefly, the pelleted cells were subjected to alkaline lysis and the DNA recovered by ion exchange chromatography by use of NucleoSpin® columns provided in the kit. Plasmid DNA was eluted in 60µl of Elution buffer (Buffer AE).

Large scale plasmid DNA preparations, maxipreps, were generated using the Endofree® Plasmid Maxiprep Kit according to the manufacturer's instructions. This yields high quality plasmid DNA without contaminating endotoxins from the *E.Coli* host. Endotoxin-free plasmid is required for mammalian cell transfection. The maxiprep DNA concentration was measured using a nanospectrophotometer as described in 2.5.

2.6.2.2 Agarose gel electrophoresis

DNA was separated by electrophoresis on a 0.8% - 2% (w/v) agarose gel (see section 2.6.1.1). Samples were mixed with one-sixth volume of 6x gel-loading buffer (Thermo Scientific, UK) and loaded into the wells of the gel. The samples were electrophoresed in 1x TBE buffer at 80V-120V for approximately 1-1.5 hours (depending on the gel percentage and appropriate fragment separation required). The bands were visualized using ultraviolet light and the estimated size of the DNA fragments made by comparison with a 1kb ladder (250-10000bp, Thermo Scientific, UK).

2.6.2.3 DNA extraction from gels

DNA bands of interest visualised in Ethidium bromide stained agarose gels were excised with a clean scalpel and DNA extracted using an ion-exchange chromatography column kit (QIAquick Gel Extraction Kit ; Qiagen, UK) according to the maufacturer's instructions. The DNA was eluted in 40 µl of Elution Buffer EB). DNA concentration was measured using a nanospectrophotometer as described in section 2.5.

2.6.2.4 DNA Sequencing

DNA was either sequenced in-house or sent externally for sequencing.

For external sequencing, a small amount of plasmid DNA was sent for Sanger sequencing at SourceBioscience along with the relevant primers.

In-house DNA sequencing was performed using the Applied Biosystems sequencing kit (Life Technologies, UK). The reaction contained: 1x BigDye® sequencing buffer, 4µl BigDye® v3.1, 0.5µl 10uM forward or reverse primer, 500ng DNA template made up to a final volume of 10µl with dH_20. The PCR thermal cycling conditions are given in Table 6. Following this, the DNA was transferred to a clean microcentrifuge tube and precipitated by adding the following; 80ul ddH20, 1.25µl of 125mM EDTA and 280ul of 95% (v/v) ethanol and was incubated for 15 minutes at RT followed by centrifugation at 14,000xg for 30 minutes. The supernatant was removed and the remaining pellet washed with 250µl of 70% ethanol. The DNA pellet was air dried and 15µl formamide added before loading onto the ABI3700 DNA sequencer.

In both cases, analysis of the results was carried out using Sequencing Analysis software v5.2 ©2004 (Applied Biosystems, UK).

Table 6: Thermal cycling conditions for DNA sequencing reaction

Step	Time	Temperature (°C)
1	1 minute	96
2	10 seconds	96
3	5 seconds	50
4	1 minute	15
5	Go to step 2 – 15x	
6	10 seconds	96
7	5 seconds	50
8	1 minute	30
9	Go to step 6 – 5x	
10	10 seconds	96
11	5 seconds	50
12	2 minutes	60
13	Go to step 10 – 5x	
14	∞	4

2.7 RNA extraction and reverse transcription

2.7.1 Materials

DEPC-treated water and other reagents described in this section are obtained from Life Technologies UK, unless otherwise stated. RNase-free water was obtained from QIAGEN.

2.7.1.1 Lysis buffer for RNA extraction

Buffer RLT (Qiagen, UK) containing 1% ß-mercaptoethanol

2.7.2 Methods

2.7.2.1 Harvesting cells for RNA extraction

The culture medium was aspirated and cells were washed in 1x PBS. Lysis buffer (2.7.1.1) was added to the cells (350µl for $<5x10^6$ or 600µl for $5x10^6$ - $1x10^7$). The cell lysate was collected using a sterile cell scraper and transferred to a 1.5ml eppendorf tube. The mixture was vortexed briefly to ensure thorough lysis.

2.7.2.2 RNA extraction

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. The cells were homogenised using the QIAshredder columns which was centrifuged at full speed for two minutes. On-column DNase-treatment was carried out using the RNase-Free DNase Set (Qiagen, UK) according to the manufacturer's instructions. Total RNA was eluted in 30µl RNase-free water, and samples were quantified using a nanospectrophotometer as described in section 2.5.

2.7.2.3 Reverse transcription and complementary DNA (cDNA) synthesis

Reverse transcription was carried out using the SuperScript™ III first-strand cDNA synthesis kit from Life Technologies, UK. Briefly 2µg of RNA (extracted from either cells or tissue) was added to 1µl 50µM oligo dT and 1µl 10mM dNTP mix made up to 10µl with RNase-free water. The components were mixed and heated at 65°C for 5 minutes, then kept on ice for 2 minutes. To the mixture, 1µl 10x first strand RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M DTT, 1µl *RNASE Out*™

and 1µl 200U/µl SuperScript III was added. The mixture was incubated at 50°C for 50 minutes and then 85°C for 5 minutes. Finally, to remove the RNA, 2U RNase H was added and incubated at 37°C for 20 minutes. 180µl of DEPC-treated water was added to give a total volume of 200µl at 10ng/µl cDNA.

2.8 Real Time quantitative PCR

Real-time quantitative PCR (qPCR) is an accurate method of simultaneous DNA quantification and amplification (Heid *et al.*, 1996). The method exploits the 5' endonuclease activity of Taq DNA polymerase to cleave a fluorescently labelled oligonucleotide probe during PCR, thereby generating a detectable signal which is quantified after each round of amplification. Using sequence-verified positive controls, real-time PCR is often combined with reverse transcription PCR to quantify messenger RNA, enabling a researcher to measure relative and/or absolute gene expression at a particular time, or in a particular cell or tissue type.

cDNA samples were amplified using an ABI Prism 7900HT sequence detection system in a final volume of 20µl containing 2x power SYBR green master mix, 4µl 10ng/µl cDNA and 0.07µM of each forward and reverse primer. PCR reactions were performed in triplicate under the following thermal cycler conditions: 95°C for 15 minutes followed by 95°C for 30 sec and 59°C for 30 sec for 40 cycles. The PCR reaction was evaluated by dissociation curve analysis to ensure that the PCR product generated were specific.

2.9 Protein Analysis

2.9.1 Materials

Reagents used in this section are purchased from Sigma, UK unless otherwise stated.

2.9.1.1 Buffers and reagents for Immunocytochemistry and Immunohistochemistry

Phosphate Buffer Saline (PBS)

19% (v/v) 0.2M monobasic sodium phosphate was added to 0.2M dibasic sodium phosphate, followed by 1.8% (w/v) NaCl to give 0.2M PBS pH7.4.
0.1M PBS pH7.4 was made by combining equal volumes of 0.2M PBS pH7.4 and ddH_2O.

Paraformaldehyde, 4% in 0.1M PBS pH7.4

This solution was prepared under the fume hood. 8% paraformaldehyde powder was made in ddH_2O and stirred for 5-10 minutes on a hot plate. 10N NaOH was added dropwise until solution became clear. Equal parts of 8% paraformaldehyde and 0.2M PBS were combined to give 4% paraformaldehyde in 0.1M PBS pH7.4

PBS-Triton (PBST)

PBST consisted of 0.1M PBS pH 7.4 with 0.25% (v/v) Triton-X-100.

2.9.1.2 Buffers for SDS-PAGE, Western Blot and Co-Immunoprecipitation

Radioimmunoprecipitation (RIPA) buffer

RIPA buffer was used to lyse cells for cytoplasmic protein extraction with the following ingredients at final concentrations in Table 7. RIPA buffer was prepared without the protease and phosphatase inhibitors and stored at 4°C. These inhibitors were only added on the day of cell lysis to give modified RIPA buffer.

Table 7: Modified RIPA buffer ingredients.

Components of RIPA buffer	**Final concentrations**
Tris HCl pH 7.4	50mM
NaCl	150mM
Nonidet P-40	1% (v/v)
Sodium deoxycholate	0.5 (w/v)
EDTA	1mM
Phosphatase inhibitors	**Final concentrations**
Sodium fluoride	1mM
Sodium vanadate	1mM
Protease inhibitors	**Final concentrations**
Aprotinin	1µg/ml
Leupeptin	1µg/ml
Pepstatin	1µg/ml
Pefablock	1mM

SDS-PAGE running buffer (National Diagnostic Ltd, UK)

SDS-PAGE buffer was purchased as a 10x concentrated solution containing 1% (w/v) SDS; 0.25M Tris-HCl pH8.3; 1.92M glycine. This was diluted with ddH_2O to give 1x SDS-PAGE running buffer.

SDS-PAGE transfer buffer

Transfer buffer contained 20% methanol in 1x SDS-PAGE running buffer.

SDS-PAGE sample buffer, Laemmli 6x

6x Laemmli sample buffer contained 40% glycerol (v/v); 50% 0.5M Tris pH 6.8 (v/v); 8% SDS (w/v); 0.004% bromophenol blue (w/v) and 2% β-mercaptoethanol.

Stock acrylamide

Stock acrylamide contained 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide (37.5:1).

SDS-PAGE gel constituents

Two different gels were required to carry out SDS-PAGE. Table 8 shows the amounts and volume of individual components required to make a stacking and a resolving gel.

Table 8: Components for an SDS-PAGE gel

Components	Stacking gel (4%)	Resolving gel (10%)
Acrylamide	11.5% (v/v)	33% (v/v)
1.5M Tris-HCl pH8.8	-	42% (v/v)
1M Tris pH6.8	6.25% (v/v)	-
10% sodium dodecyl sulphate (SDS)	1% (v/v)	1% (v/v)
Tetramethylethylenediamine, (TEMED)	0.05% (v/v)	0.066% (v/v)
10% Ammonium Persulphate (APS)	0.075% (v/v)	0.01% (v/v)

Tris- Buffered Saline (TBS), 1 x

TBS was prepared with 25mM Tris base, 150mM NaCl and 2mM KCL. pH was adjusted to 7.4 by addition of concentrated HCl.

TBS containing Tween-20 (TBST)

TBST was made by adding 0.05% (v/v) Tween-20.

Blocking buffers

TBST containing 5% (w/v) dried skimmed milk or TBST containing 5% (w/v) Bovine serum albumin (BSA).

2.9.1.3 Primary and secondary antibodies

Table 9 lists the primary and secondary antibodies used in the data chapters for carrying out immunocytochemistry, immunohistochemistry, western blot and co-immunoprecipitation.

Table 9: List of Primary and secondary antibodies used in this thesis.

Details on host species and companies from which they were purchased are provided.

GFP – Green fluorescent protein; GFAP – glial filbrillary acidic protein

Primary antibody	**Host species**	**Source**
Anti-V5	Mouse monoclonal	Life Technologies, UK
Anti- AUTS2	Rabbit polyclonal	Abcam, UK
Anti – Actin	Rabbit monoclonal	Cell Signaling, UK
Anti-GFP	rabbit polyclonal	Abcam, UK
Anti-GFAP	mouse monoclonal	Millipore, UK
Anti-Neuronal nuclei (NeuN)	mouse monoclonal	Millipore, UK
Secondary antibody	**Host species**	**Source**
Alexa Fluor 594 anti-mouse	Goat	Life Technologies, UK
Alexa Fluor 488 anti-rabbit	Goat	Life Technologies, UK
Alexa Fluor 633 anti-mouse	Goat	Life Technologies, UK
Odyssey anti-mouse 800CW	Goat	LI-COR Biosciences, UK
Odyssey anti-rabbit 680	Goat	LI-COR Biosciences, UK

2.9.2 Methods

2.9.2.1 Immunocytochemistry

Immunostaining was performed on Neuro2a cells and 293T cells. A generalised protocol is described here. The specific antibodies and dilutions used are detailed in the relevant chapters.

For the above cell lines, 14,000 cells were seeded into 1.2cm^2 chamber slides (Nunc, UK) and incubated in a 37°C with 5% CO_2, humid incubator for 2 days. For fixation, cells were rinsed in 0.1M PBS pH 7.4 before adding 4% paraformaldehyde in 0.2M PBS pH 7.4. The cells were allowed to fix for 15 minutes at RT. The cells were then washed twice in ice cold PBS. Immediately, cells were permeabilised in PBST for 10 minutes and then removed. Next, blocking buffer containing goat serum and/or BSA in PBST with 0.3M glycine (see specific blocking buffer used in relevant chapters) was added and incubated for 30 minutes. The addition of glycine will bind free aldehyde groups that would otherwise bind primary and secondary antibodies which lead to high background. The primary antibody (diluted in the blocking buffer) was then added (see relevant chapters for dilutions used) and incubated at 4°C overnight. The following day, the primary antibody was removed and washed 3 times for 5 minutes in 0.1M PBS pH 7.4. The secondary antibody was added (diluted in blocking buffer) and incubated and protected from light at RT for 1 hour. The cells were washed thrice for 5 minutes before mounting with Prolong Gold antifade reagent with DAPI (Life Technologies, UK) and then coverslipped. Images were taken using Leica TCS SP5 fluorescent microscopy (x10 and x20 objectives).

2.9.2.2 Immunohistochemistry

Brain slices (40µm thick) were made from frozen tissue sections collected from a microtome. The brain slices were washed thrice in 0.2M PBS for 5 minutes and blocked (PBST containing 1% BSA, and 1% normal goat serum) for 1 hour at RT. Primary antibodies in their appropriate blocking solutions (see relevant chapters) were added and incubated overnight at 4°C. The following day, the primary antibody was removed and the brain slices were washed in 0.2M PBS thrice for 5 minutes to remove any residual primary antibody. Sections were then incubated with the appropriate fluorescently conjugated secondary antibodies at RT for 1

hour. The secondary antibody was removed and the slices were washed in 0.2M PBS thrice to remove any residual secondary antibody. Sections were then mounted with Prolong Gold antifade reagent with DAPI (Life Technologies, UK) and coverslipped.

2.9.2.3 Protein extraction

The cytoplasmic protein extracts were prepared 72 hours post transfection from cell monolayer which was grown in 6 well plates. The cells were placed on ice, rinsed twice with ice cold PBS and then scraped into modified RIPA lysis buffer. Samples were transferred to a tube and placed on a shaker for 15 minutes at 4°C. Cell debris was then pelleted at 10000 rpm using a bench-top microfuge for 5 minutes at 4°C. The protein supernatant was transferred to a clean ependorph tube and quantified using *DC*™ protein assay (see section 2.9.2.4).

2.9.2.4 Protein quantification

Protein was quantified using the DC™ protein assay (Bio-RAD) according to the microplate assay protocol supplied by the manufacturers. Six different concentrations of BSA (2mg/ml, 1.5mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, and 0.1mg/ml) were prepared in ddH2O, each at a total volume of 5µl, to serve as a protein standard. In addition, various dilutions of each protein sample to be quantified were prepared (1:2, 1:5, and 1:10) in modified RIPA buffer, also at a total volume of 5µl. In parallel, a 'blank' sample was prepared for both protein and BSA samples for normalization purposes. Diluted protein, BSA, and blank solutions were added into wells of a fresh microtiter plate, and 25µl of reagent A', consisting of 20µl reagent S for every ml of reagent A (Bio-RAD), was then added to each well. Then, 200µl reagent B (Bio-RAD) was added to each well and the microplate was incubated for ~15 minutes on a rocker at RT. Absorbance were then read at a wavelength of 750nm using a SpectraMax® 384 Microplate Spectrophotometer (Molecular Devices, UK). Standard curves were generated using absorbance readings for the various BSA dilutions. The protein concentrations of the samples were determined using the standard curve.

2.9.2.5 SDS-PAGE and Western Blot

i) SDS-page gel preparation

Proteins were separated on one dimensional gels according to the method of Laemmli (Laemmli, 1970) using the Mini-PROTEAN® II Electrophoresis Cell gel system (Bio-Rad Laboratories). Section 2.9.1.2 details the materials used for gel preparation. A 10% resolving gel was prepared with TEMED and APS added last as they catalyse the polymerisation. Isopropanol was added to evenly align the gel. Following polymerisation of the resolving gel, the isopropanol was discarded and rinsed with ddH_2O. A 4% (w/v) stacking gel was prepared and immediately poured above the resolving gel with the comb in place and allowed to polymerise. The resolving gels constituted three-quarter of the whole gel.

ii) Sample preparation for loading and running

30µg of protein samples were added to 5x protein sample loading (Laemmli) buffer. The protein and sample buffer mixture was heated at 95°C for 5 minutes before loading. Known protein molecular weight markers – (Precision Plus Protein Standard, 10-250kD [Bio-Rad, UK]) were also run in parallel to the samples. The gels were run in 1x SDS-PAGE running buffer at 150V for 1 hour approximately.

iii) Semidry Transfer

Separated proteins were transferred from an SDS –PAGE gel to a 0.45µm pore polyvinylidene (PVDF) membrane using a Trans-Blot® Semi Dry Transfer Cell™ (BioRad). As PVDF is highly hydrophobic, it was first soaked in methanol before use. The PVDF and extra thick blot filter paper sheets were pre-soaked in transfer buffer (detailed in section 2.9.1.2). The gel was placed onto a presoasked PVDF membrane (in methanol) and sandwiched between two sheets of extra thick filter paper (presoaked in transfer buffer) and electroblotted for 1 hour at 7V. Thus, the Trans Blot Semi Dry transfer unit was in the following order: cathode, extra thick filter blot paper, acrylamide gel, membrane, extra thick filter blot paper, and anode. Following transfer, the membrane was stained with 0.2% Ponceau S dye which is a ubiquitous stain for protein in 10% acetic acid at RT for 20 minutes for visualisation of the protein bands on the membrane. The resulting red staining was washed with 10% acetic acid before antibody incubations. The Ponceau S dye was then removed, and membranes were rinsed twice in 10%

acetic acid, followed by additional rinses in ddH_2O to remove residual dye. Following confirmation of successful transfer, membranes were covered and left at RT overnight to dry in preparation for antibody staining.

iv) Antibody incubations

PVDF membranes were immersed in methanol to reduce their hydrophobicity, and then rinsed in ddH_2O. Membranes were either blocked in a solution blocking buffer as detailed in section 2.9.1.2. Blocking solution used for positive control membranes to be exposed to β-actin antibodies consisted of 5% bovine BSA, as per the manufacturer's recommendation. All membranes were incubated at RT in blocking solution for 1 hour on a rocker.

Membranes were then rinsed briefly in TBST and primary antibodies were added at individually optimized concentrations in the relevant blocking solutions (see relavant chapters for details on blocking solutions and antibody dilutions used). Membranes were incubated with primary antibodies overnight (~16 hours) at 4°C on a rocker, which was followed by three ~10 minutes rinses in TBST. Membranes were then incubated with the relevant LI-COR IRDye secondary antibodies (usually 1:5000) depending on the host of the relevant primary antibody, in the appropriate blocking solutions for 1 hour at RT on a rocker. This was followed by three rinses in TBST and then the membranes were scanned using the ODYSSEY® Infrared Imaging System (LI-COR Biosciences, UK). Blots incubated with goat anti-Mouse 800CW infrared dye were scanned at 800nm fluorescence, and blots incubated with goat anti-Rabbit 680LT infrared dye were scanned at 700nm fluorescence.

2.9.2.6 Co-Immunoprecipitation (Co-IP)

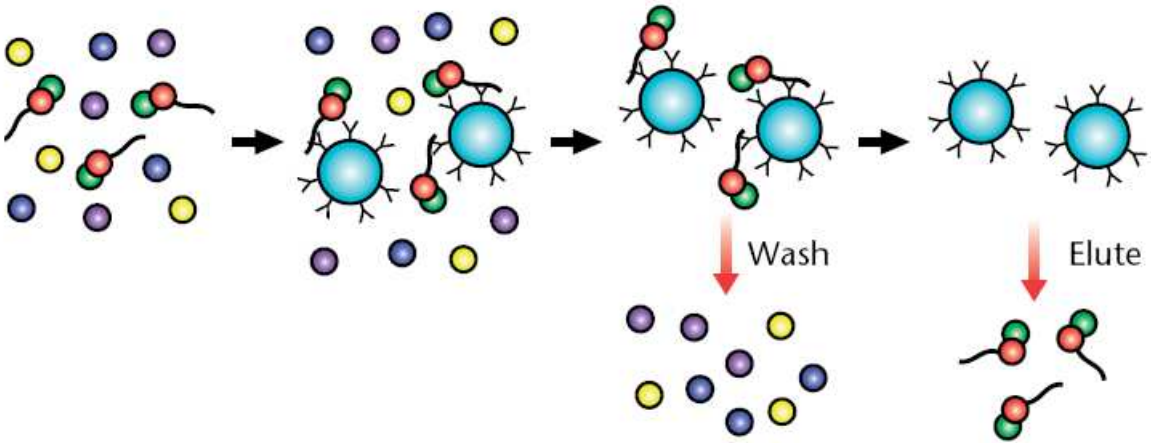
Co-immunoprecipitation (co-IP) is a classical technique for discovering PPIs by enriching a whole cell lysate for a specific protein as well as 'pull down' any other proteins that are associated with it. Basically, the idea is to capture the target protein along with its interacting partner(s), by binding to an immobilised antibody specific to the target from a mixture of proteins present within the cell (Figure 7). In brief, the antibody intended to capture the target protein is bound to protein A or G (depending on its heavy chain) which is usually covalently attached to a bead or an immobilised matrix. This binding does not affect the antibody-antigen interaction. Upon addition of the protein lysate, the antibody forms a specific immune complex with its target protein and any binding partners, resulting in a precipitate. The matrix

is separated from the lysate and washed to remove any proteins that are not specifically bound to the captured immune complex. Finally, the immune complexes are released from the immobilised matrix (by boiling) so that the target protein and its binding partners are separated for detection by SDS-PAGE, western blot analysis and mass spectroscopy (Howell *et al.*, 2006). In order to improve specificity, monoclonal rather than polyclonal antibodies are often recommended to prevent high background arising from precipitation of extraneous proteins. Tagged fusion proteins can circumvent this difficulty as the antibody targeting the tag is specific and does not cross-react with endogenous proteins. Furthermore, the use of affinity tags enables the identification of PPIs regardless of whether interacting partners bind directly or indirectly to the fusion protein; thereby potentially enabling the identification of PPIs involving multiple members of a given protein complex. This could serve to provide more comprehensive information concerning the precise molecular mechanisms within which the fusion protein participates. Furthermore, epitope-tagged proteins can often be eluted by incubation with competing peptides, or other small molecules, instead of boiling, thus preserving the integrity of the proteins captured.

Many PPIs are transient, and are characterised by small contact areas and less defined conformational changes upon binding of the interacting protein (Howell *et al.*, 2006). An advantage of co-IP is its potential to identify transient or less tightly bound interactors with weak associations due to the more physiological conditions employed. However, this method is less suitable for low abundance proteins. A frequently encountered problem is that interference from the antibody in the gel analysis of the precipitated products. The antibody present in the immune complex, contains a heavy and light chain, which may co-migrate with any potential protein binding partners. Additionally, a high level of background is often observed due to protein non-specifically interacting with the immobilised matrix. To address this problem, a pre-clearing step is carried out in which, prior to incubation with the primary antibody, the lysates is incubated with just the immobilised matrix to reduce the background.

Figure 7: Co-immunoprecipitation method

Protein of interest (red circles) with a fusion protein (black line), binding partner(s) (green circles) antibody attached to a bead (blue circles).



Recombinant fusion proteins consisting of a protein or part of it fused to a small epitope tag have been invaluable research tools in this regard. The specific epitope expression tag is coupled either at the amino- (N-) or carboxy- (C-) terminus of the protein of interest, without affecting protein function or solubility. Examples of small affinity tag protein include human influenza hemagglutinin most commonly referred to as HA (Kolodziej & Young, 1991), c-myc (Munro & Pelham, 1986), FLAG™ (Hopp *et al.*, 1988) or V5 (Southern *et al.*, 1991) tags and are recognised by specific monoclonal antibodies (Monti *et al.*, 2005). Among other applications, recombinant fusion proteins have been useful for increasing the protein yield, enhancing protein solubility, facilitating protein detection and quantification, identifying protein modification and targeting to sub-cellular compartments and extracellular localisation (e.g. secretion). Additionally, the presence of small epitope tags enables easy detection and purification of the protein of interest and is commonly used for determining protein-protein interactions which can help elucidate gene function.

In the case of AUTS2, a gene of unknown function, this thesis looks to investigate its protein binding partners by generating two expression plasmids cloned with AUTS2 in its native form and the other as V5-tagged AUTS2. Two different protocols were used depending on the antibody requirements for Co-IP as per the manufacturer's guidelines. Protein-G or Protein-A

Sepharose® resin slurry was 50% slurry in lysis buffer were obtained from Life Technologies, UK. Approximately The whole cell lysate was extracted using RIPA buffer with a cocktail of protease and phosphatase inhibitors (section 2.9.1) and quantified (section 2.9.2.4). 500µg of protein was used for each Co-IP reaction.

Anti-V5 antibody (Life Technologies, UK)

In order to minimize non-specific binding of protein to the agarose beads, cell lysates were pre-cleared by incubating 50µl Protein-G Sepharose® resin slurry per 1ml of supernatant, rocked at 4°C for 1 hour and then centrifuged for 1 minute at 10,000xg at 4°C. The supernatant was transferred to a sterile microfuge tube and placed in ice. Following this, 2µg (unless otherwise stated) of primary antibody and 50µl Protein-G or -A Sepharose® resin slurry was added to the supernatant and rocked overnight at 4°C on a rocker to facilitate the formation of immunocomplexes. The following day the supernatant was discarded and the resin was washed with 500µl RIPA lysis buffer, followed by resuspension in modified RIPA buffer to a total volume of 50µl.

Anti-AUTS2 antibody (Abcam, UK)

Protein G sepharose beads (Life Technologies, UK) were prepared by washing them three times in an excess of 1x PBS, each time followed immediately by centrifugation (~20 seconds at 10,000rpm) and removal of the supernatant. Agarose beads were then restored to a ~50% slurry in 1x PBS using a pipette tip with the end cut off to avoid disruption to the beads.

Pre-clearing was done by adding 100µl of protein G agarose beads for every ml of cell lysate. The resultant solutions were incubated at 4°C for 10 minutes on a rocker, and this was followed by centrifugation at 4°C for 10 minutes at 10,000rpm. The supernatant was transferred to a fresh 1.5ml eppendorf tube to which 5µg of anti-AUTS2 primary antibody was added and mixed gently by pipetting up and down. Tubes were incubated overnight at 4°C on a rocker to facilitate the formation of immunocomplexes.

The following day, immunocomplexes were captured by adding 50µl of washed (as described above) protein A agarose beads to each tube, followed by incubation at 4°C for 2 hours on a rocker. The agarose beads were then collected by centrifugation (~20 seconds at 10,000rpm) and the supernatant was discarded. The remaining beads/immunocomplexes were washed three times in an excess of modified RIPA buffer and then resuspended using modified RIPA buffer to a total volume of 50µl.

Finally, 10µl of 5x laemmli sample buffer containing 2% ß-mercaptoethanol was added to each coimmunoprecipitate, and samples were heated at 95°C for 5 minutes to denature the proteins ready for SDS-PAGE as per the above protocol in 2.9.2.5.

From the Western Blot analysis, if a number of other bands appear apart from the expected band for AUTS2, indicating potential interating protein partners for AUTS2, the sample would be loaded for mass spectrometry.

Chapter 3

Delineating the role of Dopamine D2 and D3 receptors in vitro and in vivo using lentiviral-mediated gene delivery

This chapter is adapted from Fernandes AR, Easton AC, De Souza Silva MA, Schumann G, Müller CP, Desrivières S. Eur J Neurosci. 2012 Apr;35(8):1344-53.

3.1 Introduction

This chapter establishes lentiviral mediated gene delivery technique in vitro and in vivo using two extensively documented genes encoding for dopamine D2 and D3 receptors (D2R and D3R), for their role in various behavioural traits including motor processes. Thus to further explore the role of D2R and D3R, lentivirus-mediated gene knockdown and overexpression was used to specifically manipulate expression levels of these genes in the rat nucleus accumbens (NAcc), a brain area important for spontaneous and induced locomotor responses. Principle findings from this chapter will contribute to delineating the role of NAcc D2R and D3R in locomotor behaviour but also the established technique will serve to conduct further investigation into the functional characterisation of AUTS2 which is covered in the chapters following the current chapter.

3.1.1 The current project and aims

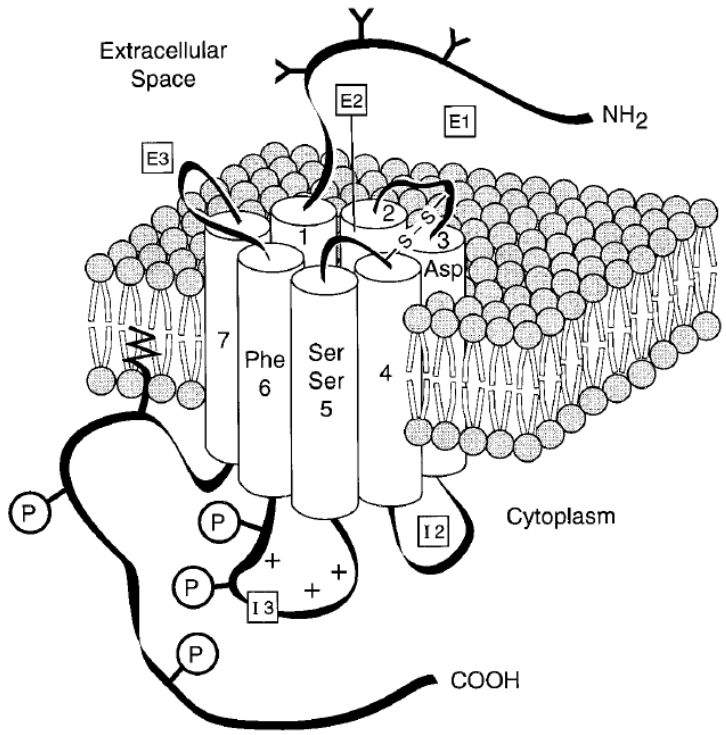
Given the limitations of using knockout mice and pharmacological agents for functional characterisation particularly in the interpretation of behavioural phenotypes, the current project applies lentiviral mediated gene delivery to address several issues with regard these techniques. As reviewed in Chapter 1, this technique not only enables the specific expression and silencing of any gene isoform *in vivo* but also allows investigation of gene function in specific brain regions on behaviour. Thus, the main aim of the present study was to establish this technique by distinguishing the individual role of D2R and D3R, two well characterised genes linked to the aetiology of several psychiatric traits and behaviours particularly locomotor behaviour. The alteration in D2R and D3R expression was thus assessed on novelty and light induced locomotor activity. For this purpose, lentiviruses were constructed which overexpressed either rat D2R or D3R. In addition, lentiviruses expressing siRNA specifically targeting rat D2R and D3R were also constructed. These viruses were tested *in vitro* and served for *in vivo* gene transfer, by means of stereotaxic injections into the rat NAcc, a brain area important for motor behaviour. Findings from this chapter will provide a validation of lentiviral mediated gene delivery *in vitro* and *in vivo* which can then be used to unravel the role of *Auts2 in vitro* and *in vivo*.

3.1.2 Dopamine receptors

The effects of DA are mediated by five different receptors that have common structural characteristics. These dopamine receptors (DRs) belong to the G-protein-coupled receptor family. Member of this family contain a glycosylated extracellular N-terminus, a seven transmembrane domain forming the hydrophobic core of the receptors, three intracellular loops which differ in length among the different receptors and an intracellular C-terminal stretch (Figure 8). Based on their structural and functional properties, the dopamine receptors are divided into two subgroups (Brown & Makman, 1972; Kebabian & Calne, 1979): 'D1-like' comprising D1R and D5R which are highly homologous (Dearry *et al.*, 1990; Sunahara *et al.*, 1990) and 'D2-like' consisting of D2R, D3R and D4R (Bunzow *et al.*, 1988; Sokoloff *et al.*, 1990; Strange, 1993a). D1-like receptors are coupled to stimulatory Gα-proteins which enhance the activity of adenylyl cyclase and induces cAMP production and D2-like receptors inhibit the activity of adenylyl cyclase by coupling to inhibitory Gi- proteins (Neve *et al.*, 2004) and thus reduces cAMP production. It is thought that many of the effects of D_2–like receptors may be mediated by βγ subunits (Herlitze *et al.*, 1996; Ikeda, 1996).

Figure 8: The dopamine receptor structure.

The five dopamine receptors consist of a seven transmembrane domains and are coupled to G proteins (either stimulatory or inhibitory) which mediate various signalling pathways. This figure is taken from (Missale et al., 1998)



Both D2R and D3R belong to the D2-like receptor family. D2R is approximately 52% homologous to D3R sharing similarity in the transmembrane domains. D2R exists as two alternatively spliced isoforms: short ($D2R_S$) and long ($D2R_L$), which differ by 29 amino acids in the third intracellular loop (Monsma *et al.*, 1989; Usiello *et al.*, 2000). Since this loop is thought to play an important role in receptor coupling, the resulting splice variants may possess different functional roles (Missale *et al.*, 1998). Both variants share the same distribution pattern although the short isoform is less abundantly expressed (Giros *et al.*, 1989; Monsma *et al.*, 1989). Although both isoforms inhibit adenyly cyclase, $D2R_S$, has higher affinity than $D2R_L$ (Dal Toso *et al.*, 1989; Montmayeur & Borrelli, 1991). It has been reported that protein kinase C (PKC) signalling modulates $D2R_S$ and $D2R_L$ signalling with a selective inhibitory effect on the $D2R_S$ –mediated response (Liu *et al.*, 1992). As of yet, there are no compounds that discriminate between the two variants. Some reports suggest that the $D2R_S$ functions as the

presynaptic receptor, whereas the $D2R_L$ is a postsynaptic receptor and $D2R_S$ is expressed to a greater extent in presynaptic receptors than in postsynaptic receptors (Lindgren *et al.*, 2003; Jomphe *et al.*, 2006). Further supporting the role of the $D2R_S$ isoform being a presynaptic receptor are studies from knockout mice of D2R (both short and long isoforms) and $D2R_L$ alone which suggest that $D2R_S$ mediates autoreceptor responses of dopaminergic neurons and $D2R_L$ is responsible for postsynaptic responses (Baik *et al.*, 1995; Kelly *et al.*, 1998; Usiello *et al.*, 2000).

The rat D3R contains 446 amino acids compared to the human protein, which is made up of 400 amino acids (Giros *et al.*, 1989; Sokoloff *et al.*, 1990). Splice variants also exists for D3R but only in mice (Fishburn *et al.*, 1993). The isoform differ by 21 amino acids in the third intracellular loop. They have similar pharmacological profiles and have the same distribution profile in the brain with the long isoform being predominantly expressed.

3.1.3 Distribution of D2R and D3R in the brain

The expression profiles of D2R and D3R differ in the brain, which suggests they play distinct roles. D2R is broadly distributed in the brain and is highly expressed in the striatum, olfactory tubercle, and the core of the NAcc. D2R is also found in the prefrontal cortex, amygdala, substantia nigra pars compacta, ventral tragmental area where it is expressed by the dopaminergic neurons. Immunohistochemical analysis shows that D2R is present in the medium spiny neurons of the striatum where they are more concentrated in the spiny dendrites and spine heads (Missale *et al.*, 1998).

On the other hand, D3R has a distinctive pattern of localisation in the brain, expressed preferentially in the limbic brain regions displaying highest expression levels in the islands of Calleja (Diaz *et al.*, 1995). It is also present at high levels in the NAcc, olfactory tubercles and the bed nucleus of the stria terminalis in the mesocorticolimbic system which influences motor function, cognition and motivation (Sokoloff *et al.*, 1990; Bouthenet *et al.*, 1991; Xu *et al.*, 1997). In the NAcc, D3R is mainly post-synaptically located (Diaz *et al.*, 1995). Due to the preferential expression of D3R in these brain regions, D3R was suggested as a therapeutic target for the negative and cognitive symptoms of schizophrenia, drug addiction, and Parkinson's disease (Joyce & Milian, 2005; Newman *et al.*, 2005; Joyce & Millan, 2007). The distribution of D3R in the human brain, although not very well studied, is by and large similar to that observed in the rat, where increased levels of D3R mRNA is detected in the NAcc and

islands of Calleja and comparatively low levels observed in the anterior caudate and putamen (Landwehrmeyer *et al.*, 1993).

3.1.4 The neural circuitry of the ventral striatum and the nucleus accumbens

The striatum and the NAcc are prominent structures of the basal ganglia that modulate different physiological functions such as motor behaviour, reward, motivation and learning (Mogenson *et al.*, 1980; Nicola *et al.*, 2000; Zahm, 2000). The intrastriatal circuitry consists of medium spiny GABAergic neurons that are the origin of two major striatal efferent pathways (called direct and indirect pathways), which connect the striatum to the other basal ganglia nuclei including the NAcc. These neurons transmit information carried by the DA fibres originating in the substantia nigra and by the cortical glutamatergic neurons (Nicola *et al.*, 2000). The interplay between DA and glutamate is what drives proper motor functions.

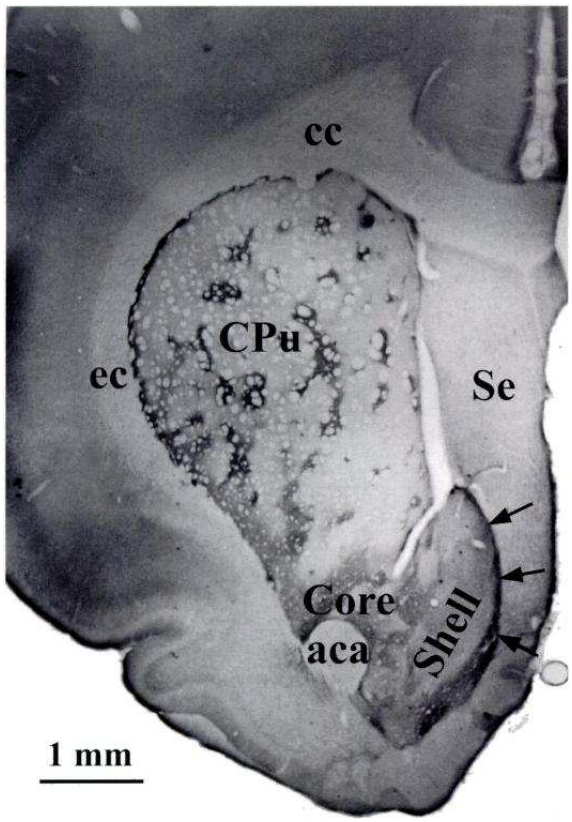
The NAcc receives excitatory innervation from limbic structures such as the prefrontal cortex, hippocampus, and amygdala (Swanson & Cowan, 1975; Sesack & Pickel, 1990; Odonnell & Grace, 1995). Its main efferents innervate the pallidum, striatum, mediodorsal thalamus, prefrontal, including cingulate cortex and, as mentioned above, mesolimbic dopaminergic areas. The NAcc attains, thus, a central position between limbic as well as mesolimbic dopaminergic structures, basal ganglia, mediodorsal thalamus and prefrontal cortex. Together with the ventral striatum, the accumbens receives a strong input from the VTA and the dorsal tier of the substantia nigra (Groenewegen *et al.*, 1999; Zahm, 2000). Degeneration of dopaminergic cells in the substantia nigra leads to loss of DA in the striatum, which is the underlying cause of Parkinson's disease (Albin *et al.*, 1989). Dysregulation of dopaminergic signaling in the striatum and NAcc can also lead to the development of several other common neuropsychiatric disorders, including Tourette's syndrome, schizophrenia, and drug addiction (Nemeroff & Bissette, 1988; Koob & Nestler, 1997).

The NAcc is subdivided into a central 'core' region, which is surrounded on its medial and ventral sides by a peripheral 'shell' (Herkenham *et al.*, 1984; Zaborszky *et al.*, 1985) (Figure 9). The (medial) shell region is predominantly innervated by the dopaminergic cells of the ventral tegmental area (A10 cell group) whereas the core region is predominantly innervated by the dopaminergic cells of the substantia nigra (A9 cell group) (Groenewegen *et al.*, 1999). The core region contains a larger proportion of relatively small cells with high concentrations of D1- and D3-receptors (Heimer, 2000). Within the NAcc, information is transmitted from shell to core.

The NAcc has been demonstrated to play an important role in locomotor behaviours. This was demonstrated from observations that lesions in the accumbens cause a reduction in spontaneous and induced locomotion (Kelly & Iversen, 1976; Fink & Smith, 1980). Furthermore, injections of DA or DA agonists into the NAcc induce hyperlocomotion (Pijnenburg & van Rossum, 1973; Jackson *et al.*, 1975). Similarly, strong locomotor activation is elicited by microinjection of cocaine, a potent blocker of presynaptic DA reuptake in the NAcc (Delfs *et al.*, 1990). Accumbal dopamine is not only involved in mediating motor function but also in processes involved in reward and reinforcing effects of drugs of abuse (Arias-Carrion *et al.*, 2010).

Figure 9: An anatomical localisation of the nucleus accumbens core and shell in a rat brain coronal section taken from (Heimer *et al.*, 1997).

The Nacc is sometimes referred to as a ventral extention to the striatum; CPu – caudate putamen, Se – septum, cc – corpus callosum, ec – external capsule, aca – anterior commissure



3.1.5 D2R and D3R regulation of locomotor behaviour

Injection of dopamine in the rat NAcc was shown to increase locomotor activity (Pijnenburg & van Rossum, 1973), suggesting a role for dopamine receptors in the control of this behaviour. Most of our current knowledge of D2R and D3R is obtained from studies using agonists, antagonists and knockout mice (Beaulieu & Gainetdinov, 2011).

For D3R, there is substantial literature on the effect of pharmacological ligands stimulating and inhibiting D3R on motor activity. Systemic administration of the D3R agonists, 7-Hydroxy-2-(di-npropylamino) tetralin (7-OH-DPAT) and PD 128907, dose dependently decreased motor activity in mice (Gyertyan and Saghy 04;Millan et al 04). As seen in D3R knockout mice, numerous findings show that D3R antagonists enhance spontaneous locomotion in rodents (Waters et al 93a;Waters et al 93b;Klingpetersen et al 95;Sautel et al 95;Clifford and Waddington 98;Manzanedo et al 99;Carr et al 02;Gyertyan and Saghy 04;Millan et al 04;Pritchard et al 07). It must be noted that low doses of the putative dopamine D3R antagonist, PNU 99194A, increased locomotor activity while higher doses suppressed locomotor activity (Gendreau et al 97). However, pharmacological approaches provide only an indication of the role of both D2R and D3R in locomotor function as results generated from these studies can be inconclusive since pharmacological agents vary in their affinities to and specificities for a given receptor (Heidbreder *et al.*, 2005; Joyce & Milian, 2005). Further complications are raised by the recent recognition that D2R and D3R can heterodimerize, or even form functional complexes with D1R, which prevents straightforward interpretation of the actions of dopamine agonists and antagonists (Maggio & Millan, 2010). D2R agonists induce locomotor activity (Eilam *et al.*, 1991; Hoffman & Wise, 1992; Ralph & Caine, 2005). While, lack of D2R attenuates spontaneous locomotion (Braun et al 97;Millan et al 04;Stuchlik et al 07;Huang et al 10). This is in line with knock out studies; the first study using D2R knockout mice reported a significant decrease in locomotor behaviour in the open field and no rearing behaviour (Baik *et al.*, 1995), concordant with later studies (Kelly *et al.*, 1998; Aoyama *et al.*, 2000; Fowler *et al.*, 2002). This phenotype is further exacerbated in mice mutant for both D2R and D3R (Jung *et al.*, 1999). On the other hand, mice lacking the D3R only, exhibit hyper-locomotion (Accili *et al.*, 1996; Xu *et al.*, 1997). Although these findings indicate an important role of both these receptors in locomotor behaviour, results from the knockout studies are questionable due to genetic compensatory regulatory mechanisms, developmental

effects, and lack of regional specificity (Xu *et al.*, 1997; Boulay *et al.*, 1999a; Usiello *et al.*, 2000; Wang *et al.*, 2000; Barik & de Beaurepaire, 2005).

This thesis also looks at the role of dopamine receptors in light- induced locomotor activity in rats. Sensory stimuli such as light and sound can induce locomotor behaviour in humans and animals which may indicate behaviour of exploration or fear. This sensorimotor processing or gating can be studied in rodent models. Presently, the most widely used model of sensorimotor gating is prepulse inhibition (PPI). This paradigm relies on the use of sound (prepulse), usually bursts of noise typically with the intensity of around 110dB (Hoffman & Ison, 1980) being more of an aversive stimulus generating a reflexive startle response in rodents (Geyer *et al.*, 2001; Swerdlow *et al.*, 2001). PPI is the attenuation of a startle response by a preceding non-startling sensory stimulus. Deficits in such behaviour has been seen in people under the influence of drugs, schizophrenia and Alzhiemer's patients (Braff *et al.*, 2001; Swerdlow *et al.*, 2008). D2R and D3R have been investigated for their role in PPI (Swerdlow *et al.*, 1990; Swerdlow *et al.*, 1992; Wan *et al.*, 1994; Varty & Higgins, 1998; Swerdlow *et al.*, 2001; Ralph-Williams *et al.*, 2002; Zhang *et al.*, 2006; Zhang *et al.*, 2007; Swerdlow *et al.*, 2009; Chang *et al.*, 2010). Another stimulus used for sensorimotor gating is light. Light-induced locomotor activity is a paradigm for sensorimotor processing and provides a reliable method of generating a behavioural response to a visual stimulus in the rat (Pum *et al.*, 2009b). It uses a low intensity range light (less than or equal to 440lx) which is not aversive and triggers exploratory behaviour observed by an increase in locomotor activity as this visual stimulation appears to be novel and provides some incentive salience for the animal. It has been previously shown that visual stimulation increases locomotor activity and rearing behaviour (Muller *et al.*, 2007a; Pum *et al.*, 2009a; Pum *et al.*, 2009b). This basic behavioural process which may either lead to habituation of the stimulus response or the learning of associations of the stimulus with subsequent events. Importance of this behaviour has been shown in the investigation of the effect of cocaine on attention and responsiveness to sensory stimiuli in rodents which would be relevant for patients of drug abuse. Reports have also shown that on-off visual stimulation works as a reinforcer in rodents (Roberts *et al.*, 1958; Hughes, 2007; Olsen & Winder, 2009) as well as in primates (Blatter & Schultz, 2006). In light of the abovementioned findings, it is important to investigate the contribution of t both these receptors in the control of sensory stimuli induced response. As of yet, there are no studies looking at the specific role of D2R and D3R in light-induced locomotor activity.

3.2 Methods and Materials

3.2.1 Materials

3.2.1.1 Cloning plasmids LeGO - iG2 and LeGO-G

cDNAs and short hairpin oligos were cloned into Lentiviral Gene Ontology (LeGO) vectors (Weber *et al.*, 2008; Weber *et al.*, 2010). These lentiviral expression vectors allow efficient transfer and expression in many different cell types including haematopoietic stem cells. Derived from the third generation lentiviral vectors LentiLox7.3, which do not encode any viral proteins but contain *cis*-active elements for packaging, reverse transcription, and integration, they are able to incorporate 9kb of foreign sequences LeGO –G (Figure 10) and LeGO – iG2 (Figure 11) vectors contain an enhanced Green Fluorescent Protein (eGFP) marker while LeGO – C (Figure 10) vector contains a red fluorescent marker called Cherry (Weber et al., 2008).

Figure 10: LeGO-G and LeGO-C lentiviral vectors for gene silencing.

This figure is taken from (Weber et al., 2008). The LeGO-G and LeGO-C vectors, contains a retroviral enhancer/promoter of spleen focus-forming virus (SFFV), which allows a broad and high expression pattern for expression of the reporter gene eGFP (enhamnced Green Flouresecent Protein) or Cherry, while the U6 promoter drives the expression of the shRNA cloned into the Multiple cloning site (MCS) using the HpaI and XhoI restriction sites. Other features include rev-responsive element (RRE); self-inactivating-long-terminal repeat (SIN-LTR); Woodchuck hepatitis virus post-transcriptional regulatory element (wPRE) and central polypurine tract (cPPT).

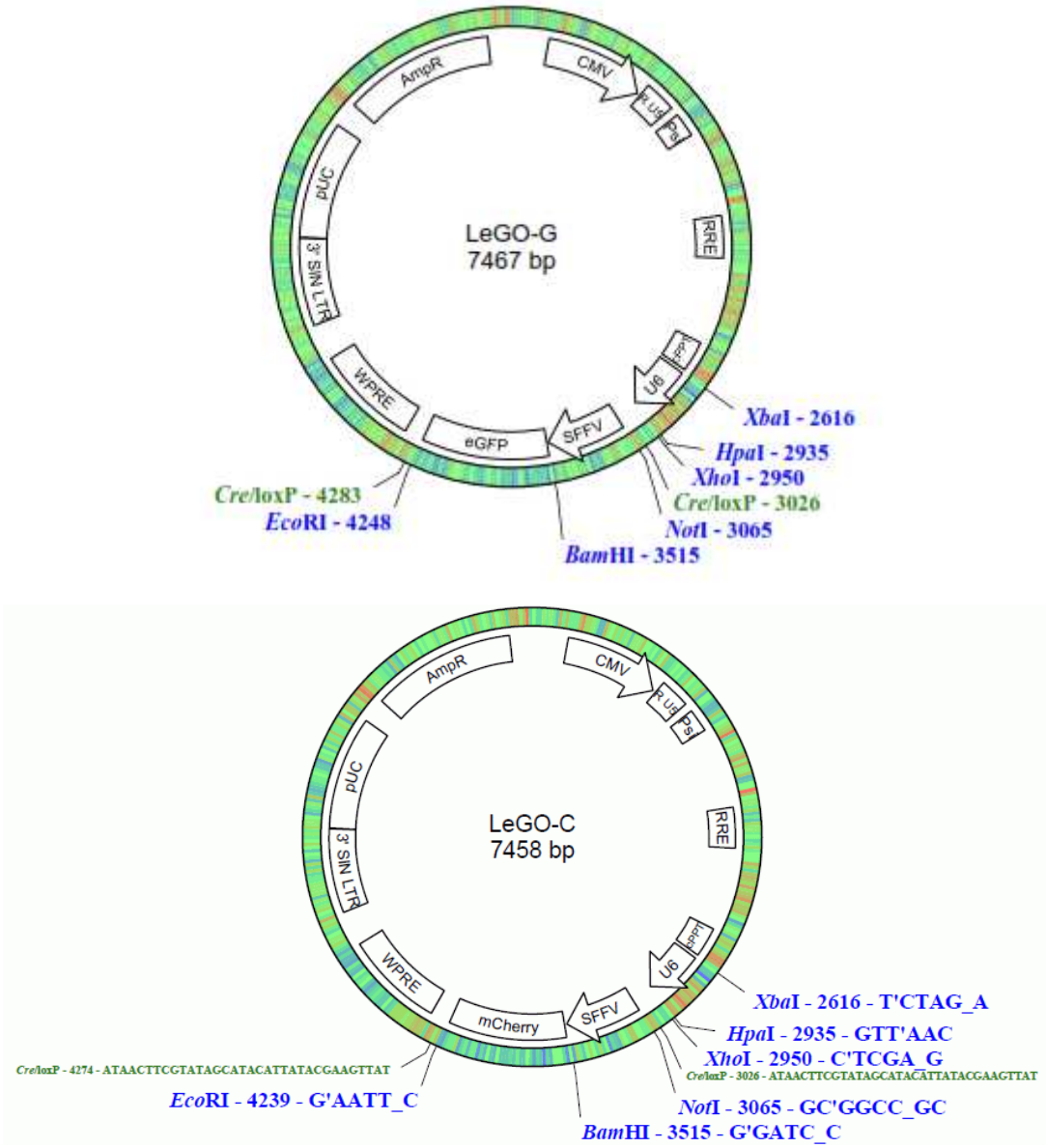
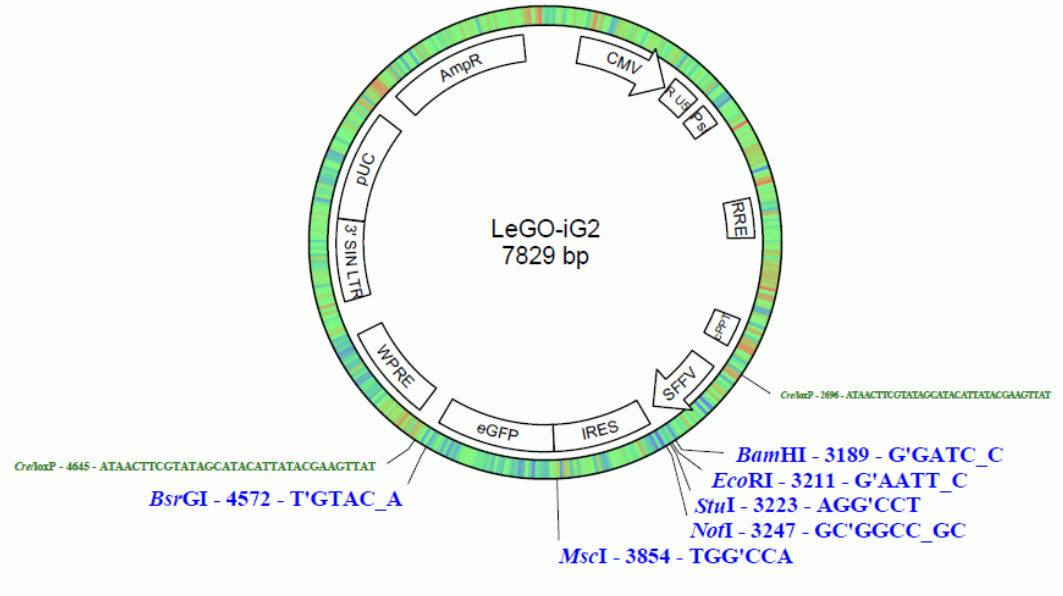


Figure 11: The lentiviral overexpression vector LeGO-iG2.

This figure is taken from (Weber et al., 2008). *The overexpression vector LeGO-iG2, contains an SFFV promotor, which allows a broad and high expression of the transgene in the MCS. Both D2R and D3R were cloned into the EcoRI and NotI site in the MCS. This is followed by an encephalomyocarditis virus internal ribosome entry site (IRES), to facilitate the expression of the marker gene, GFP by the same promotor.*



3.2.2 Construction of D2R and D3R overexpression vectors

3.2.2.1 PCR Amplification of D2R and D3R

RNAs isolated from rat brain lysates (section 2.7) were reverse-transcribed using the SuperScript® III First-Strand Synthesis System (Life Technologies, UK) as described in section 2.7. The cDNA for the D2R short isoform (D2RS) and D3R cDNAs (GenBank accession no. NM_012547 and NM_017140, respectively) were then amplified by PCR using the following forward and reverse primers (restriction sites are underlined):

Table 10: Primers used to amplify D2R and D3R for cloning into the LeGO- IG2 vector.

Forward and reverse primer sequences contain an EcoR1 and a Not1 restriction site respectively (underlined).

Primer	Sequence 5' > 3'
D2R-F	TGCCGGAATTCATGGATCCACTGAACCTGTCCTGGT
D2R-R	AAGGAAAAAAGCGGCCGCTCAGCAGTGCAAGATCTTCATG
D3R-F	TGCCGGAATTCATGGCACCTCTGAGCCAGATAAGCA
D3R-R	AAGGAAAAAAGCGGCCGCTCAGCAGGACAGGATCTTGAG

The forward primers contain an *EcoRI* restriction site (underlined) followed by the start codon and the 5' rat cDNA-specific sequence; the reverse primers contain the 3' rat cDNA-specific sequence, a stop codon and a *NotI* restriction site (underlined). The PCR reaction consisted of: 40ng rat whole brain cDNA, 1x PCR buffer (Life Technologies, UK), 0.2mM dNTPs, 0.5µM *forward primer*, 0.5µM *reverse primer*, 7.5mM $MgCl_2$, 1U Platinum Taq (Life Technologies, UK) and dH_20 in a final volume of 50µl. The thermal cycling conditions are detailed in Table 11.

Table 11: Thermal cycling conditions for touchdown PCR to amplify rat D2R and D3R.

Step	Time	Temperature (°C)
1	9 minute	95
2	30 seconds	95
3	30 seconds	56
4	1 minute 30 seconds	72
5	Go to step 2 - 40 times	
6	10 minutes	72
7	∞	4

3.2.2.2 Agarose gel electrophoresis

The PCR reactions were run by electrophoresis on a 0.8% (w/v) agarose gel as detailed in section 2.6.2.2, and the expected bands excised, gel-purified (as detailed in 2.6.2.3) and stored at -20°C until further use.

3.2.2.3 Restriction enzyme digest

The D2R and D3R PCR products were digested using the EcoRI and NotI restriction enzymes, which cleave DNA at the 5' region of both sets of primers. A typical reaction contained 2 units of each enzyme /1µg DNA, 1x Buffer EcoRI, 1x BSA, made up to a final volume of 20µl with ddH_20. The reaction mix was incubated at 37°C for 1 hour to allow complete digestion, followed by 20 minutes at 65°C to inactivate the restriction enzymes. Fragments were purified using the QIAquick PCR purification kit (QIAGEN, Crawley, UK) as detailed in section 2.6.

The LeGO-iG2 lentiviral expression vector was also digested using the EcoRI and NotI restriction enzyme in the conditions described above. In addition, in order to prevent re-circularization of the plasmid after restriction enzyme digestion, the phosphate groups at the ends of the linear vector were removed. Thus digested vectors were dephosphorylated by the addition of 1U of Antarctic phosphatase/1µg DNA and 1x Antarctic phosphatase buffer to the restriction digest and samples incubated at 37°C for 30 minutes. Following this, the enzyme was heat-inactivated for 20 mins at 65°C. Finally, to check for complete digestion of the plasmid, 200ng of the plasmid DNA was gel

electrophoresed (as detailed in section 2.6.2.2. Plasmid DNA was purified using the QIAquick PCR purification kit (QIAGEN, Crawley, UK).

3.2.2.4 Ligation of the PCR products into the LeGO-iG2 lentiviral expression vector

DNA ligation reactions were carried out using a 1:3 DNA molar ratio for the digested, dephosphorylated vector and digested insert DNA respectively. Typically, 50ng of vector and 150ng insert DNA was mixed with 1x T4 DNA ligase buffer and 1U T4 DNA ligase (NEB, UK) in a total reaction volume of 10µl. In parallel, control ligations were set up containing digested, dephosphorylated plasmid with no insert plus ligase and used as background to check for vector DNA restriction quality. The ligation reactions were incubated overnight at 16°C. 5µl of the ligation reaction was transformed into a 50µl aliquot of MAX EFFICIENCY® Stbl2™ cells (Life Technologies, UK as described in section 2.4. Colonies were picked and minipreps of the precultures were prepared in order to screen for positive clones; i.e. bacterial clones that contained the vector plus insert.

3.2.2.5 Screening for positive clones

In order to screen for positive bacterial clones, minprep DNA was digested with EcoRI and NotI. The restriction digest consisted of 1U of each enzyme in 1x Buffer EcoRI, 1x BSA and 16 µl of miniprep plasmid DNA. The reaction was incubated for 1 hour at 37°C and run on 0.8% w/v agarose gel. A digest of the empty vector was also carried out and run alongside the miniprep digests for comparison. The molecular weight of the D2R and D3R inserts were 1332 bp and 1341 bp, respectively.

3.2.2.6 DNA sequencing of positive clones

DNA sequencing was performed using the Applied Biosystems sequencing kit. The reaction contained: 1x BigDye® sequencing buffer, 4µl BigDye® v3.1, 0.5µl 10uM forward or reverse primers (Table 12). 500ng DNA template made up to a final volume of 10µl with dH_20. The PCR thermal cycling for the sequencing reaction is detailed in Table 13. Following the PCR reaction, the DNA was transferred to a clean microcentrifuge tube and precipitated by adding 80ul ddH_20, 1.25µl of 125mM EDTA and 280ul of 95% (v/v) ethanol. The DNA was precipitated for 15 minutes at RT and centrifuged at 14,000 x g for 30 minutes. The supernatant was discarded and the

remaining pellet washed with 250µl of 70% ethanol. The DNA pellet was air-dried and 15µl of formamide added before loading onto the ABI3700 DNA sequencer. Analysis of the results was carried out using Sequencing Analysis software v5.2 ©2004 (Applied Biosystems, UK).

Table 12: Primers used to sequence D2R and D3R positive clones.

Primers were designed at around 300bp within the sequence in order to cover the entire sequence length. These were also used for sequencing.

Primer	Sequence 5' > 3'
D2R-F	CGTGGGTTGTCTACCTGGAG
D2R-R	TATACCGGGTCCTCTCTGGG
D3R-F	ACGTTTTTGTCACCCTGGAT
D3R-R	CCTGTGGCATCTGACAGAAA

Table 13: PCR thermal cycling for the DNA sequencing reaction.

Step	Time	Temperature (°C)
1	1 minute	96
2	10 seconds	96
3	5 seconds	50
4	1 minute	15
5	Go to step 2 – 15x	
6	10 seconds	96
7	5 seconds	50
8	1 minute	30
9	Go to step 6 – 5x	
10	10 seconds	96
11	5 seconds	50
12	2 minutes	60
13	Go to step 10 – 5x	
14	∞	4

3.2.3 Construction of D2R and D3R shRNA recombinant lentiviruses

3.2.3.1 D2R and D3R shRNA design

The small interfering RNA sequences, targeting the coding domain of the *Drd2* (both isoforms) and *Drd3* transcripts were designed using the siRNA Retriever software (http://cancan.cshl.edu/RNAi_central/). The sense target sequence is shown in Table 14.
For D3R, two shRNAs were designed since a combination of the two gave a higher knock down efficiency than either used on its own. The target sequences for D3R listed have been used previously (Bahi *et al.*, 2005) with highly efficient silencing of the receptor *in vitro* and *in vivo*. The oligos were ordered with the addition of a 5'phosphate and PAGE purified.

Table 14: Sense target sequence of shRNAs targeting D2R and D3R

Gene	Sense target sequence 5' > 3'
D2R	ATCCACTGAACCTGTCCTGGTACGATGAC
D3R (1)	TGGTGAGCCTGGCTGTGG
D3R (2)	TGGAGTCTGGAATTTCAG

These sequences were used to synthesize complementary oligonucleotides for vector-based small hairpin RNA (shRNA) expression using the following design:
For D2R: Sense oligo: 5' TG - (28 nt, sense oligo) CAAGCTTC (28 nt, antisense oligo) TTTTTTC. Antisense oligo: complement of sense with additional nucleotides at 5' end to generate XhoI overhang (Brummelkamp *et al.*, 2002).
For D3R: Sense oligo: 5' T - (18 nt, sense oligo) – (TTCAAGAGA) – (18 nt, antisense oligo) – TTTTTTC. Antisense oligo: complement of sense with additional nucleotides at 5' end to generate XhoI overhang.

3.2.3.2 Annealing shRNA oligos

The sense and antisense oligos, (60pmoles of each) were annealed in buffer containing 100mM potassium acetate, 30mM HEPES-KOH pH 7.4 and 2mM magnesium acetate, by incubation at 94°C for 4 minutes, 70°C for 10 minutes followed by a temperature decrease of 0.1°C/minute until to 4°C.

3.2.3.3 Restriction enzyme digest of LeGO-G and LeGO-C plasmids

LeGO-G and LeGO-C plasmids described in section 3.2.1.1 (Weber *et al.*, 2008), were digested using the HpaI and XhoI restriction enzymes. A typical reaction contained 2U of each enzyme /1µg DNA, 1x Buffer EcoRI, 1x BSA made up to a final volume of 20µl with dH_20. The reaction mix was incubated at 37°C for 1 hour to allow complete digestion, followed by 20 minutes at 65°C to inactivate the restriction enzymes. To prevent re-circularisation of the vector, a dephosphorylation step was carried with 1 unit of Antarctic phosphatase/1µg DNA and 1x Antarctic phosphatase buffer and incubated at 37°C for 30 minutes followed by enzyme inactivation for 5 mins at 65°C. Finally, to check for complete digestion of the plasmid, 200ng of the plasmid DNA was gel electrophoresed. Plasmid DNA was purified using the QIAquick PCR purification kit (QIAGEN, Crawley, UK).

3.2.3.4 Ligation of annealed shRNA into the LeGO-G plasmid vector

DNA ligation reactions were carried out using a 1:1 DNA molar ratio for the digested, dephosphorylated vector and annealed oligos. Typically, 30fmoles of vector and 30fmoles of annealed oligos was mixed with 1x T4 DNA ligase buffer and 1U T4 DNA ligase (NEB, UK) in a total reaction volume of 10µl. In parallel, control ligations were set up containing digested, dephosphorylated plasmid with no insert plus ligase and used as background to check for vector DNA restriction quality. The ligation reactions were incubated overnight at 16°C. 5µl of the ligation reaction was transformed into a 50µl aliquot of MAX EFFICIENCY® Stbl2™ cells (Life Technologies, UK), as described in section 2.4. Individual colonies were picked and grown for precultures and subsequently minipreps were carried out for plasmid DNA extraction.

3.2.3.5 Screening for positive clones

miniprep DNA digestion was carried out using XhoI and XbaI to screen for the presence of an insert. The reaction consisted of 1x Buffer 4, 1x BSA, 1 unit of each enzyme and 18µl of miniprep DNA. Both enzyme restriction sites were present in the multiple cloning site of the vector and positives were analysed in comparison to empty vector. The expected sizes were as follows: For empty vector: 449bp + 7kb; for vector + insert: 509bp + 6.9kb. Due to this very small difference, the digest were run on a 2% gel (w/v) for 1.5 hours at 60V.

3.2.3.6 DNA sequencing of ligated LeGO-G vector

Cloned inserts were sequenced to verify the integrity of each construct following the sequencing protocol described in 2.6.2.4, using the following primer which recognises the U6 promotor sequence in the vector: CGCACAGACT TGTGGGAGAA GCTCG

3.2.4 Production and titration of lentiviruses

Lentiviruses were prepared by co-transfection (calcium phosphate co-precipitation) of one of the gene transfer plasmids (carrying either D2R or D3R cDNAs, or shRNAs against D2R or D3R or the empty plasmids) together with the packaging plasmids into HEK-293T cells, as described previously (Jankiewicz *et al.*, 2006; Vafaizadeh *et al.*, 2010). This protocol was previously developed and optimised in the lab. The transfection was carried out according to the following protocol: The day before transfection, $5x10^6$ cells were plated in complete medium in 10cm dishes to obtain 80% confluency. The following day, three hours prior to transfection, the medium was replaced with 5ml fresh culture medium. For each dish to be transfected, the reaction mix was prepared in a final volume of 500µl with 10µg gene transfer plasmid; 5µg pRSV-Rev; 10µg pMDLG/pRRE; 3.5µg pMD2.G (plasmids obtained from Open Biosystems, Thermo Scientific, UK) with 50µl 2.5mM $CaCl_2$. 500µl of 2x Hepes Buffer Saline (HBS) pH7.12 was slowly added in a dropwise manner whilst applying air pressure to generate a bubbling effect to mix the DNA/$CaCl_2$/HBS solutions. This was left at RT for 30 minutes. Immediately prior to the transfection, 1µl of 100mM chloroquine was added to the 5ml culture medium followed by 1ml of the calcium phosphate precipitated DNA and left overnight at 37°C with 5% CO_2 humid incubator. The following day, the medium was replaced with 10ml complete medium. Lentiviruses-containing supernatants were collected 48 and 72 hours after transfection, pooled, filtered through 0.22µm pore nitrocellulose and stroed at -80°C. For lentiviral concentration, viral supernatants were ultracentrifuged as detailed in section 2.3.2.3. General titres obtained using this protocol ranged from 1.0-2.0 $x10^9$ TU/ml.

3.2.5 Transduction of 293T cells with lentiviruses either expressing or silencing D2R and D3R

In vitro expression analysis was carried out to assess the effectiveness of the lentivirus constructs. In order to a) quantify the expression level of D2R and D3R; b) investigate knock down efficiency of shRNAs; c) test specificity of shRNAs, HEK293T cells were infected with lentiviral supernatants as follows:

For Drd3 expression: 1) empty vector; 2) D3R only; 3) D3R + shD3R; 4) D3R + shD2R
For Drd2 expression: 1) empty vector; 2) D2R only; 3) D2R + shD2R; 4) D2R + shD3R

The day before the infection, $1x10^5$ HEK293T cells were plated per well in six-well plates. On the day of infection, the lentiviral supernatants were added to the cells in a total volume of 3ml medium with a final concentration of 0.04mg/ml Polybrene (Sigma, UK). The plates were centrifuged for 90 minutes at 2500rpm at RT and then incubated for 2.5 hours at 37°C in a 5% $C0_2$ incubator after which the medium was replaced and incubated back for 72 hours. The cells were lysed for RNA extractions using the QIAGEN RNeasy Mini Kit (QIAGEN, UK), followed by cDNA synthesis (as described before in 2.7.2.3).

3.2.6 Quantification of D2R and D3R transcripts levels

cDNAs were amplified by quantitative PCR, with β−actin as an internal control. The primers are listed Table 15. qPCR was carried out as in section 2.8.

Table 15: qPCR primers for rat D2R, D3R and human β−*ACTIN*

Primer	Sequence 5' > 3'
D2R-F	TCCCAGCAGAAGGAGAAGAA
D2R-R	ATTCAGGATGTGCGTGATGA
D3R-F	GATGGTGGTCATTGTGCTTG
D3R-R	TGTAAAGCTCTGGGGACACG
β-*ACTIN*-F	GCTCGTCGTCGACAACGGCTC
β-*ACTIN*-R	CAAACATGATCTGGGTCATCTTCTC

3.2.7 Stereotaxic surgery and lentivirus injection

The stereotaxtic surgeries in the rat NAcc and lentiviral injections were carried out by Alanna Easton and Dr Christian Müller.

Rats were anesthetized using 0.2 ml/100g (intra - peritoneally) of a mixture of Ketamine (100mg/ml), Medetomidine (1mg/ml) and sterile water (analgesic treatment: 5 mg/kg Rimadyl, subcutaneously). They were then placed in a Kopf stereotaxic frame and an incision in the scalp was made. The skin was retracted to expose the skull. Animals were bilaterally injected with 2 µl concentrated lentiviral stock (1.1-1.2 × 10^9 TU/ml) into the NAcc (coordinates vs. bregma/skull surface: AP +1.8, ML ± 2.2, DV -7.4; (Paxinos & Watson, 2007)) via an injection cannula by using a microinfusion pump (flow-rate: 0.2 µl/min). Animals received a D2R- (n=7) or D3R over-expressing lentivirus (n=7) or an empty vector control injection (n=8) or lentiviruses expressing shRNAs targeting either D2R (n=12), or D3R (n=10) or the corresponding empty vector (n=8). Following the injection, the cannula was left in place for 5 min to allow for diffusion of the lentivirus particles, and then retracted in steps of 0.5 mm/ 5min for 1 mm before being finally removed. The holes in the skull were closed with dental cement and the wound was sutured and disinfected. Animals were allowed to recover for one week and were handled daily. There were no losses of animals due to e.g. animal health or injection misplacement. Animals were tested 2 weeks or 2 months after injection (see Project timeline in 3.2.8). Since the expression of cellular markers as well as the behavioural results was similar between animals with various test latencies, data were collapsed for analysis.

3.2.8 Behavioural testing

The animal handling, behavioural testing and analysis was carried out by Alanna Easton and Dr. Christian Müller.

3.2.8.1 Open field

In the open field test, locomotor activity and anxiety-related behaviour were measured on day 1 of testing. On day 2 habituation learning was assessed. Testing was carried out in an open-field (40 x 40 x 39 cm) of a light-beam fitted TruScan system (Coulbourn Instruments, Allentown, USA). Each animal was tested in the open field for 20 min on 2 consecutive days. For open field testing the animal was placed into the middle of the open field, facing away

from the experimenter (Figure 12). Locomotor activity was measured as distance moved and rearing, as the number of times the animal lifted both forepaws from the ground, in blocks of 5 min (Figure 13). To measure anxiety-related behaviour in the open field (Prut & Belzung, 2003; Pum *et al.*, 2009a), the arena was virtually subdivided in a 30 x 30 cm centre and a periphery. Centre entries were measured as the number of times the animal entered the centre. Centre time was the time that the animal spent in the centre. Habituation learning was assessed by comparing locomotor activity and rearing from day 1 and day 2 (Thiel *et al.*, 1999) (see timeline of project below).

Figure 12: Open field paradigm

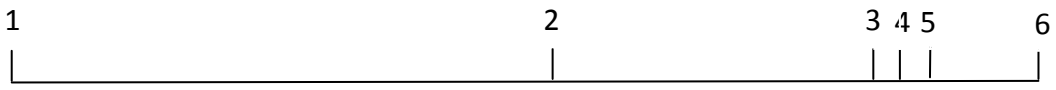
A commonly used test to measure general locomotor activity, anxiety and rearing behaviour in rodents using automated equipment.

Figure 13: Rearing behaviour in rats

The rat on the left is disolaying rearing behaviour which is when the animal stands in its hind legs lifting its paws in order to explore the environment for threat or explore a novel environment.

Timeline of project

1. Generation of lentiviral plasmids for overexpressing and knocking down D2R and D3R and lentiviral production. Testing shRNA for knock down efficieny in vitro. Large scale lentiviral production.
2. Stereotaxic injections in rats.
3. 2 weeks or 4 weeks post injection: Behavioural testing for novelty-induced locomotion 'Day 1'
4. Behavioural testing for novelty-induced locomotion 'Day 2'. On this day, light-induced locomotor behaviour was measured (refer to the next section for more details),
5. Animal culling and brain extraction and fixation
6. Brain slicing and Immunostaining



3.2.8.2 Light-induced activity

Light-induced locomotor activity is a paradigm for sensorimotor processing and provides a reliable method of generating a behavioural response to a visual stimulus in the rat (Pum *et al.*, 2009b). It uses a low intensity range light (less than or equal to 440 lux) which is not aversive and triggers exploratory behaviour observed by an increase in locomotor activity as this visual stimulation appears to be novel and provides some incentive salience for the animal.

After animals were habituated to the open field with an illumination of 32 lux on day 2 of the testing for 20 min, light-induced activity was assessed (see Timeline of project above). During a subsequent 20 min test interval, 10 light stimuli (constant white light) with 30 sec duration each were presented, distributed randomly over the 20-min period. The light was a constant white light of 420 lux. Locomotor activity and rearing were measured automatically by the TruScan light beam system.

3.2.9 Extraction of rat brains and fixation

The brains were removed, fixed and sliced by Alanna Easton.

After behavioural testing, (i.e. either 2 or 4 weeks post lentiviral injection), animals were deeply anesthetized with 0.5 ml Nembutal (containing 0.6 mg/ml Pentobarbital; Sanofi, France) and transcardially perfused with 0.5% sodium nitrite (in 0.1 M phosphate buffered saline pH7.4, PBS) for 3-5 minutes, and 4% paraformaldehyde (in 0.1M PBS) solution for 15 min. Brains were removed and stored overnight in 4% paraformaldehyde solution, moved for 15 min to a 10% sucrose (in 0.1M PBS) solution and finally stored in a 30% sucrose + 0.05% NaN3 (in 0.1M PBS) solution for atleast 24 hours.

3.2.10 Histological analysis

Immunohistochemical staining was performed as per section 2.9.2.2, with primary and secondary antibody dilutions as shown in Table 16. Images were taken using Leica TCS SP5 confocal microscopy (x20 and x100 objectives).

Table 16: Primary and secondary antibodies used in histological analysis of rat brains

Primary antibody	Primary antibody dilution	Secondary antibody	Secondary antibody dilution
Anti-GFP	1: 1000	Alexa Fluor 488	1:1000
Anti- NeuN	1:1000	Alexa Fluor 633	1:1000
Anti-GFAP	1:1000	Alexa Fluor 633	1:1000

3.2.11 Statistics

Quantification of D2R and D3R expression in HEK293T cells*:* Data were expressed as mean ± SEM and normalised to the values obtained from cells infected with Lenti-D2R and Lenti-D3R. To verify a between treatment difference, a one-way ANOVA with factor treatment was used. To test for treatment differences, pre-planned comparisons were conducted using the independent sample *t*-test.

Behavioural analysis: data were expressed as mean ± SEM. To assess the effects of gene overexpression or knockdown on behaviour, two-way ANOVAs with the factors treatment and time were used. To test for group differences at single time points, pre-planned comparisons were conducted using *t*-tests (Ramsey, 1993). An alpha level of <0.05 was considered statistically significant.

3.3 Results

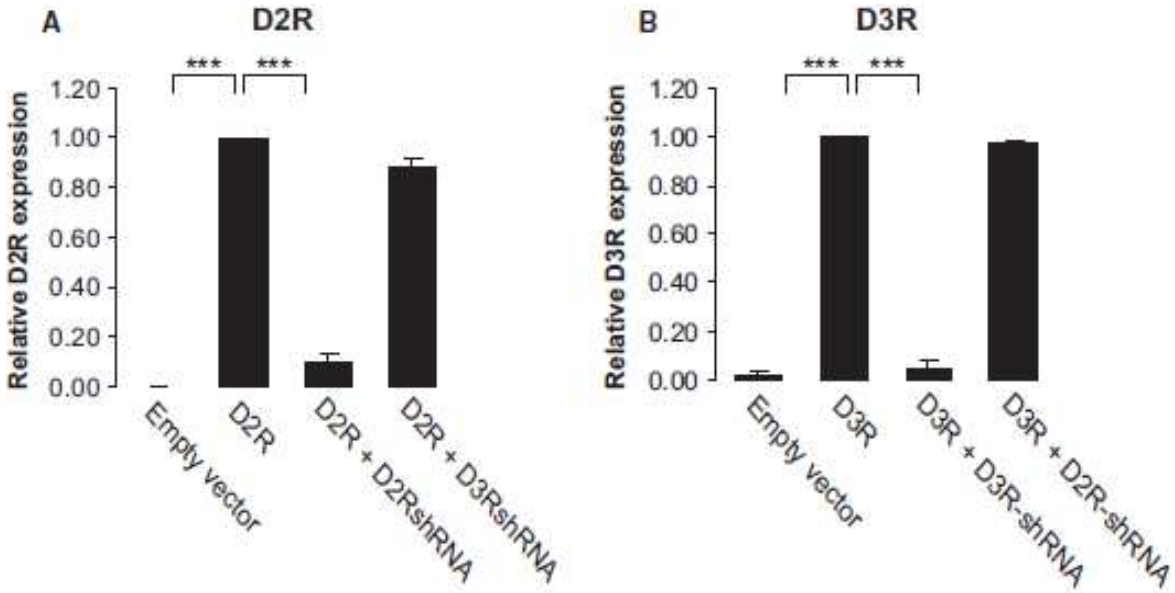
3.3.1 Generation of lentiviruses altering expression of D2R and D3R

For *in vitro* experiments, Human Embryonic Kidney, (HEK) 293T cell line were used, which does not endogenously express D2R and D3R. In order to test the knockdown efficiency of the shRNAs *in vitro*, this cell line was co-infected with the overexpression and knockdown constructs. Thus to distinguish the lentivirus efficiency during transduction *in vitro*, LeGO- iG2 was used to overexpress our gene of interest (green) and LeGO – C (mCherry; red) was used to clone the shRNA . In parallel, the same shRNAs were cloned in LeGO – G (GFP; green) which would be used for *in vivo* experiments. Since the lentiviruses overexpressing and silencing D2R and D3R would be injected separately into the animals, this was simply a preference as GFP fluorescence is generally easily observable than mCherry (for example when determining the level of lentiviral spread in the rat brain slices; see next section). The lentiviruses of the same titre were used (1.1×10^9 TU/ml) for all experiments.

As shown in Figure 14, infection of cells with lentiviruses with D2Rs or D3R cDNAs led to high expression of the corresponding transcripts. Co-infection with lentiviruses expressing a selective shRNA led to drastic reduction of the targeted receptor, with no effect on the expression of the other, related receptor. There was significant effect between groups on D2R ($F_{4,10}$= 824.43, p<0.001) and D3R expression ($F_{4,10}$=1006.05, p<0.001). Post- hoc analysis showed significant knockdown of D2R by D2R-shRNA (p<0.001) and D3R by D3R-shRNA (p<0.001). No significant differences in D2R expression were found when comparing cells expressing D2R alone and cells expressing D2R + D3R-shRNA (p=0.102; Figure 14A). Similarly, no difference in D3R expression was observed when comparing cells expressing D3R alone and cells expressing D3R + D2R-shRNA (p>0.05; Figure 14B). These results demonstrate the efficiency and specificity of the D2R- and D3R targeting lentiviral constructs.

Figure 14: Effects of D2R- and D3R-targeting lentiviral constructs on D2R (A) and D3R (B) expression *in vitro*.

*HEK293T cells were infected with lentiviruses carrying an empty vector, D2RS encoding vector (D2R) or D3R-encoding vector (D3R), alone or in combination with lentiviruses encoding shRNAs targeting D2R (D2R-shRNA) or D3R (D3R-shRNA), as indicated. Quantification of mRNA levels was performed by real-time quantitative PCR (mean + S.E.M from 3 independent experiments), ***p<0.001, ANOVA followed by t-test vs D2R or D3R overexpression.*

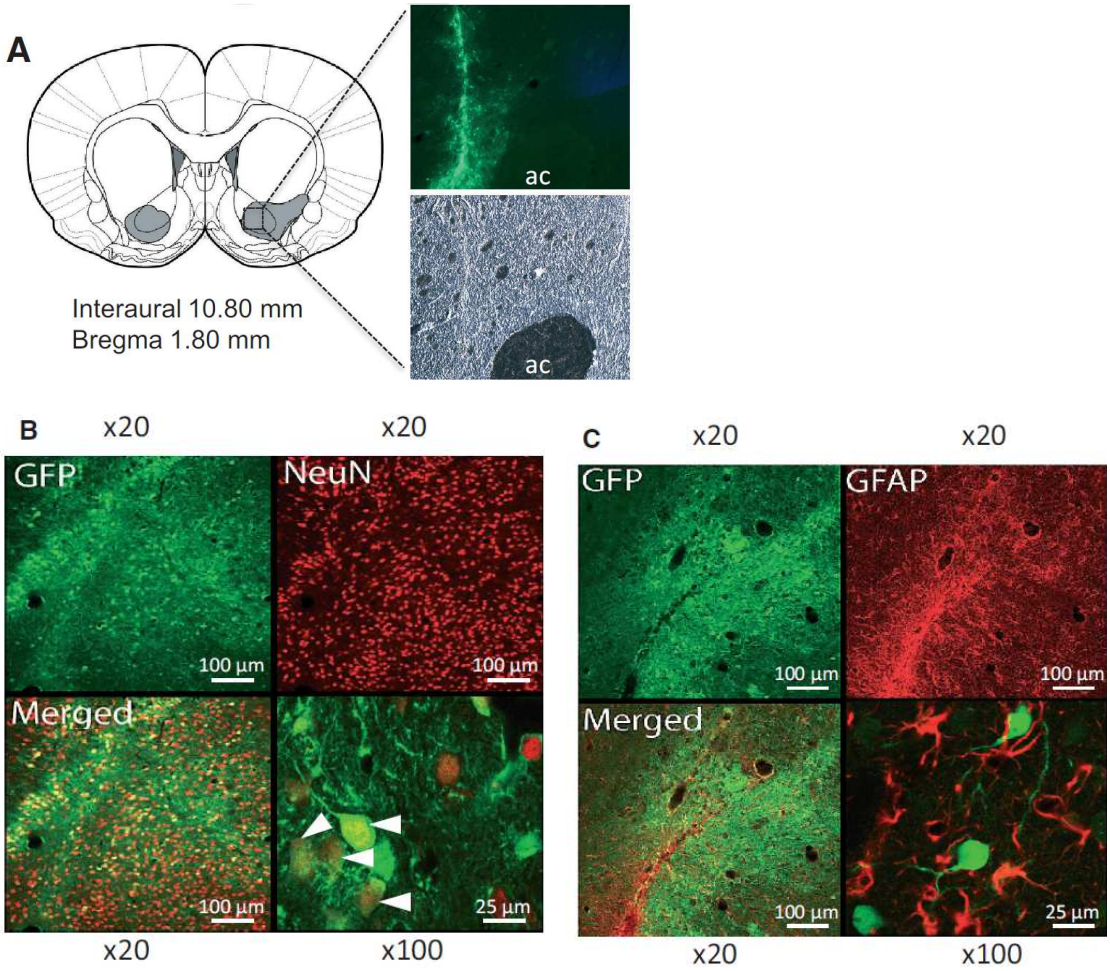


3.3.2 Stereotaxic lentivirus injections and immunohistochemical analysis

To investigate the function of D2R and D3R in the NAcc, lentiviruses were bilaterally injected in the brain of rats and the injection sites confirmed by Immunohistochemical analysis. Staining of brain sections for the lentivirus-associated GFP marker demonstrated proper targeting of the NAcc (Figure 15, A). Co-localisation of GFP with the neuronal marker NeuN (Figure 15, B), but not with the glial marker fibrillary acidic protein (GFAP) (Figure 15, C), indicated that NAcc neurons were primary targets of the lentiviruses. This having been verified, the consequences of interfering with D2R and D3R expression with lentiviruses on behaviours were investigated (see next section).

Figure 15: Immunohistochemical analysis of lentiviral-mediated gene delivery in the NAcc

A) Graphical representation of the NAcc areas in which lentiviral constructs were expressed Grey areas in both hemispheres represent cumulative immunochemistry staining for GFP from brains of all injected animals. ac – anterior commissure B) High levels of neuronal infection in the NAcc (bar=100µm): Green staining is green fluorescent protein (GFP) marker from virus. Red staining shows neuronal nuclei (NeuN). The white arrowheads in the magnified image (bar=25µm) shows GFP-positive and NeuN-positive neurons in the NAcc. C) Infection of glial cells in the NAcc was not detected, at x20 magnification (bar = 100µm). Green staining is GFP from virus. Red staining shows the astrocyte marker, glial fibrillary acidic protein (GFAP). The x100 magnified image (bar = 25µm) shows no co-localisation between the GFP-positive cells and glial cells. This image and other used for analysis presented here are taken from rats 4 weeks after lentiviral injections.



3.3.3 Reduction of novelty-induced locomotor activity by either NAcc D2R or D3R knockdown

The effect of NAcc D2R and D3R manipulation was investigated on locomotor behaviour in rats as well as rearing behaviour (standing on hind legs usually to survey the environment for possible threats or novelty). Both these behaviours were measured using the open field paradigm as described in the methods sections above.

Rats injected with lentiviruses overexpressing D2R or D3R in the NAcc displayed no effect on locomotor activity on either test day 1 (when the environment is novel) or day 2 (when habituation of the environment has occured) (Figure 16) compared to empty vector controls. The figure shows that there was an effect of time on both days (day 1: $F_{3,57}$=71.89, p<0.0001; day 2: $F_{3,57}$=51.38, p<0.0001), i.e. over the course of the session on each day, locomotor activity decreased which indicated a within-trial habituation, however there was no effect of neither treatment nor interaction between the three groups (p>0.05). Similarly, overexpression of D2R or D3R in the NAcc had no effect on rearing behaviour. There was also a within-trial habituation of rearing behaviour on both test days (day 1: $F_{3,57}$=18.74, p<0.0001; day 2: $F_{3,57}$=13.04, p<0.0001), but no effect of treatment nor interaction (p>0.05) between groups.

Knockdown of either D2R or D3R in the NAcc significantly reduced spontaneous locomotor activity on day 1, but not on day 2 (Figure 16). There was an effect of treatment on day 1 ($F_{2,30}$= 5.65, p=0.008), an effect of time ($F_{3,90}$= 130.35, p<0.0001), and a treatment x time interaction ($F_{6,90}$= 2.62, p=0.022). The D2R knockdown group showed a significant reduction in locomotor activity on day 1 (when the open field arena represented a novel environment) in all four test intervals (p=0.01; p=0.001; p=0.021; p=0.024). The D3R knockdown group showed a significant reduction in locomotor activity on day 1 in the last three test intervals (p=0.005; p=0.011; p=0.008) and a tendency in the first one (p=0.07). These effects were not observed on day 2 anymore indicating that this effect only occurs in a novel environment. While there was still an effect of time ($F_{3,90}$= 48.74, p<0.0001) for each group, where there a decrease of locomotor behaviour over time as expected. There was no significant effect of treatment or interaction on day 2 (p>0.05) between any of the groups.

The knockdown of D2R and D3R in the NAcc had no significant effect on rearing behaviour on day 1 or 2. There was a within-trial habituation of rearing behaviour on both test days (day 1: $F_{3,66}$= 51.89, p<0.0001; day 2: $F_{3,90}$= 22.17, p<0.0001), but no effect of treatment or interaction (p>0.05) between any of the groups.

As changes in locomotor activity in the activity arena may reflect anxiety (Prut & Belzung, 2003), a possible contribution of anxiety to the behavioural changes observed above was investigated, using the elevated plus maze test. Neither D2 nor D3 receptor over-experssion had an effect on time spent in open arms or entries to it (p>0.05). However, number of entries, but not time spent in the closed arms was significantly increased in D3 over-expressing animals (p<0.0047) suggesting at least a small anxiogenic effect in this paradigm (Figure 17). The knock down of D3 receptors in the Nac increased tendentially the time spent in the closed arms of the maze (p<0.074), thus, suggesting a small anxigenic effect. Neither time nor entries to the open arms were changed, nor was the number of closed arm entries (p>0.05). Indeed due to technical issues, we could not prove that D2R and D3R were indeed overexpressed or knocked down in vivo (see discussion) so the above result must be interpreted with some caution.

Figure 16: The effects of NAcc D2R and D3R overexpression and knockdown on locomotor activity and rearing behaviour.

*Testing was carried out on day 1 (novel environment) and day 2 (familiar environment), (mean ± SEM), **p < 0.05, *p < 0.01, ANOVA followed by t-tests vs. empty vector (EV) control. Overexpression of D2R and D3R showed no differences in neither locomotor nor rearing behaviours on both days. D2R and D3R knockdown in the NAcc led to decreased locomotion and a tendency for decreased rearing on day 1 but not on day 2.*

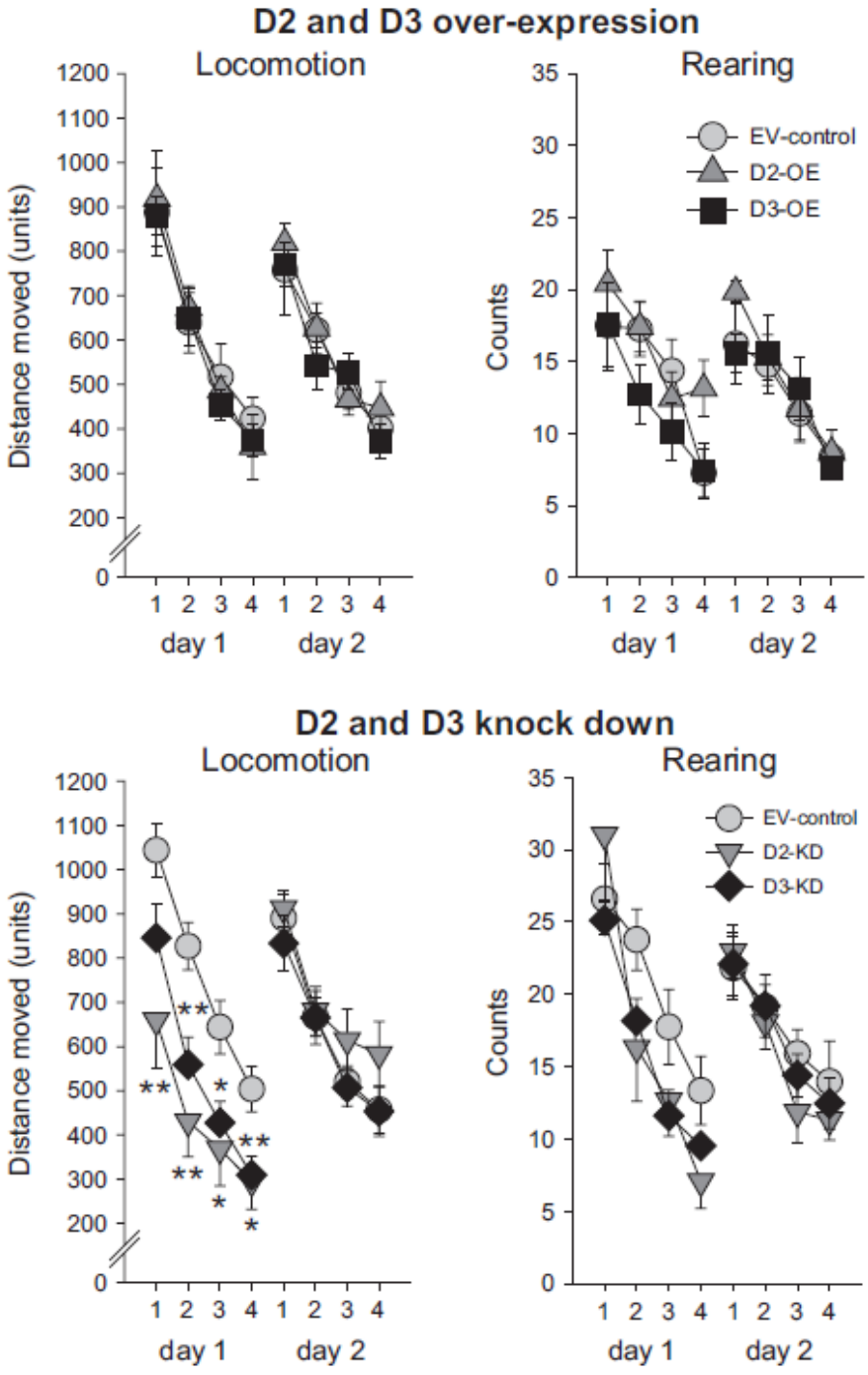
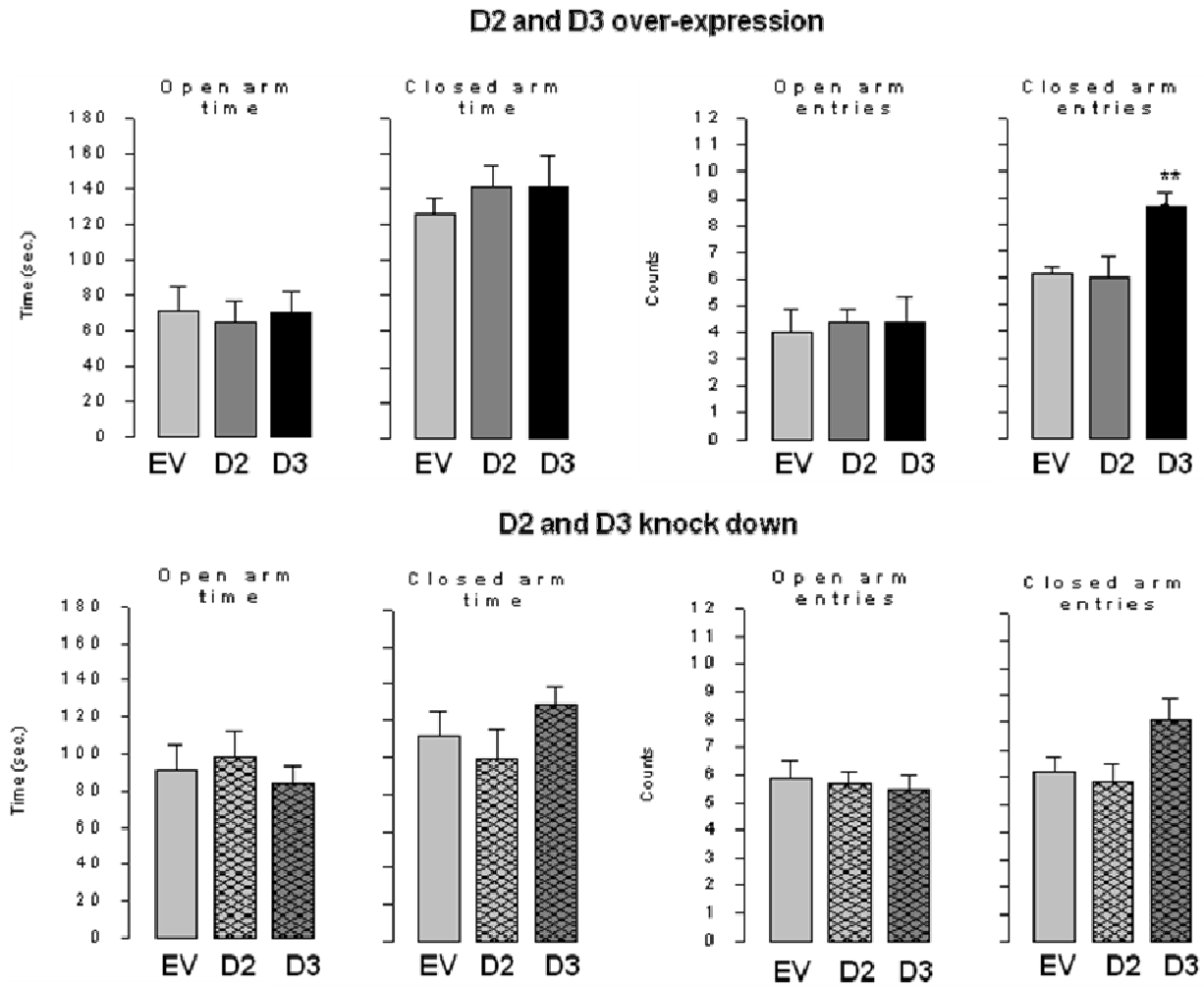


Figure 17: **Effect of nucleus accumbens D2R and D3R over-expression and knock down on anxiety-related behaviour in the elevated-plus-maze test.**

*Increased open arms entries and time spent indicate less anxiety and vice versa. Neither D2R nor D3R silencing and over-experssion had an effect on time spent in open arms or entries to it. With the exception of D3R overexpression where the , number of entries, but not time spent in the closed arms was significantly increased. Mean ± S.E.M, **p<0.005, ANOVA followed by t-tests vs. empty vector (EV) control.*



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3.3.4 Light-induced activity is controlled by NAcc D2R and D3R

As D2R and D3R knockdown demonstrated a decrease in novely-induced locomotor behaviour, it would be interesting to see how manipulation of these genes would have an effect on light induced locomotor behaviour. In a well habituated environment, the presentation of a new unconditioned stimulus, such as a bright light, can induce exploratory activity measured as an increase in locomotion and/or rearing (Muller *et al.*, 2007a; Pum *et al.*, 2008). It has been previously shown that light-induced exploratory activity is highly stable during repeated testing (Pum *et al.*, 2009b). Thus, the current chapter aimed at investigating the possible involvement of D2R and D3R in light-induced exploratory activity.

In this paradigm, once the animal was habituated to its environment (in this case the open field box), a non-aversive light stimulus was presented. Locomotor and rearing behaviour was measure for 10 minutes at 5-minute interval. Not surprisingly, as seen in Figure 18, the presentation of a light stimulus resulted in an increase in locomotor activity and rearing behaviour in control animals injected with empty vectors, but there was also an increase in light-induced activity in the animals injected with D2R- and D3R- expressing lentiviruses only in the first 5 mins of presenting the light stimulus (time: $F_{2,45}$= 16.91, $p < 0.0001$ in the first 5-min interval vs. baseline (before presentation of the light stimulus) ; for empty vector, p= 0.001; D2R, p = 0.026; and D3R, p = 0.011However, compared to the empty vector controls light-induced locomotion was significantly lower in animals injected with D2R-expressing lentiviruses ($p < 0.027$; Figure 18,A). While, injection of D3R-expressing lentiviruses had no significant effect ($p > 0.05$) on locomotion. Rearing activity was also increased in all groups; (time: $F_{2,45}$= 22.54, $p < 0.0001$ in the first 5 min interval compared to baseline. Empty vector, p = 0.001; D2R, p = 0.0031; D3R, $p < 0.0001$). When comparing with the empty vector control, the light-induced increase in rearing behaviour was not affected by D2R or D3R receptor overexpression ($p > 0.05$; Figure 18,B).

In the case of knockdown, as expected the presentation of a bright light stimulus resulted in an increase in locomotor activity in control animals injected with empty as well as DA-receptor-shRNA expressing lentiviruses (time: $F_{2,81}$ $p < 0.0001$, first 5 min interval vs. baseline; Figure 19, A). Light-induced activity was altered in rats injected with D2R- or D3R-shRNA-expressing lentiviruses (Figure 19,A) as there was a significant effect on locomotion ($F_{2,27}$=23.42, p< 0.0001). Group comparisons revealed that the injection of lentiviruses expressing D2R-shRNA

in the NAcc significantly increased light-induced locomotion (p = 0.002) compared to control. This is supported by attenuation of this behaviour for D2R overexpression described above. Contrary to the behaviour seen for D2R, the knockdown of D3R in the NAcc reduced light-induced locomotion (p = 0.002) compared to empty vector control.

The presentation of the light stimulus had a significant effect on rearing behaviour ($F_{2,27}$= 3.47, p = 0.045) in all thee groups compared to baseline (time: $F_{2,81}$= 23.21, p < 0.0001, first 5 min interval vs. baseline; empty vector, p = 0.0019; D2R, p < 0.0001; D3R, p = 0.0065). However, there were no significant effect of neither D2R nor D3R knock down on light-induced rearing compared to the empty vector controls although D3R knockdown resulted in a tendency for diminished rearing behaviour (p = 0.056; Figure 19,B).

Figure 18: **The effect of nucleus accumbens D2R and D3R overepression on light-induced locomotor activity (mean + S.E.M.).**

*Left figures show the time course of the light-induced activation (hatched bar – time of light- stimulation ;BI - baseline. Bar graphs (right panels) show genotype differences when activity was collapsed over 10 minutes of stimulation) *p>0.05, ** p>0.01, ANOVA followed by t-tests vs. Empty vector (EV) control.*

A) The left panel shows that locomotor activity upon introduction of a light stimulus was increased in all three groups including EV. However, this behaviour was significantly reduced in animals injected with D2R-expressing lentiviruses vs. EV (p< 0.027) while, D3R-expressing lentiviruses had no significant effect (p>0.05; right panel). B) Rearing activity was also increased in all three groups after the light stimulus was introduced (left figure). Although, the light-induced increase in rearing behaviour was not significantly different by D2R or D3R receptor overexpression (p> 0.05) compared to EV.

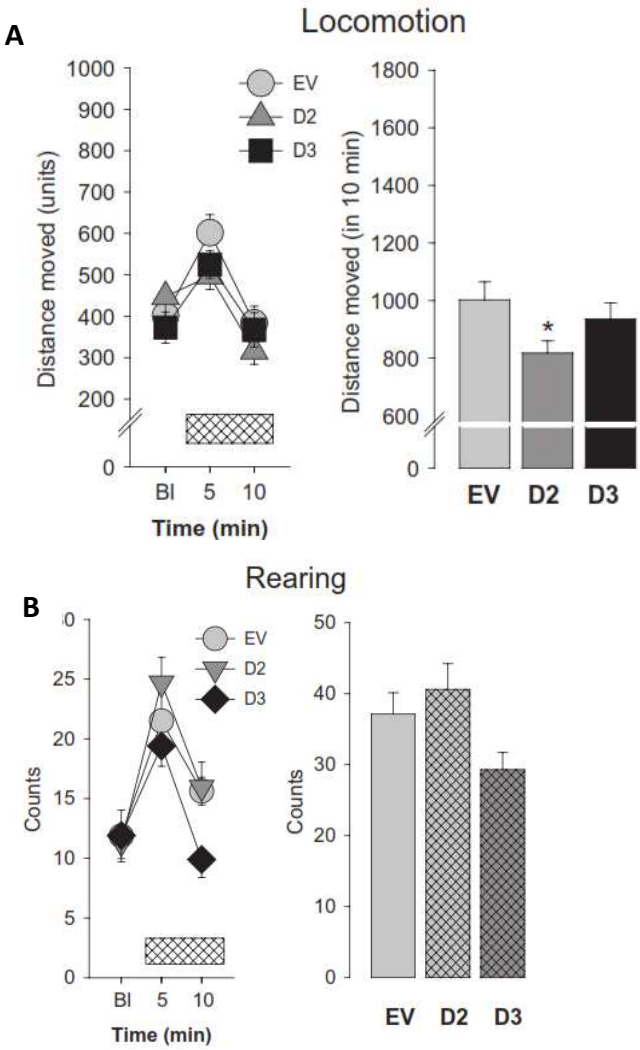
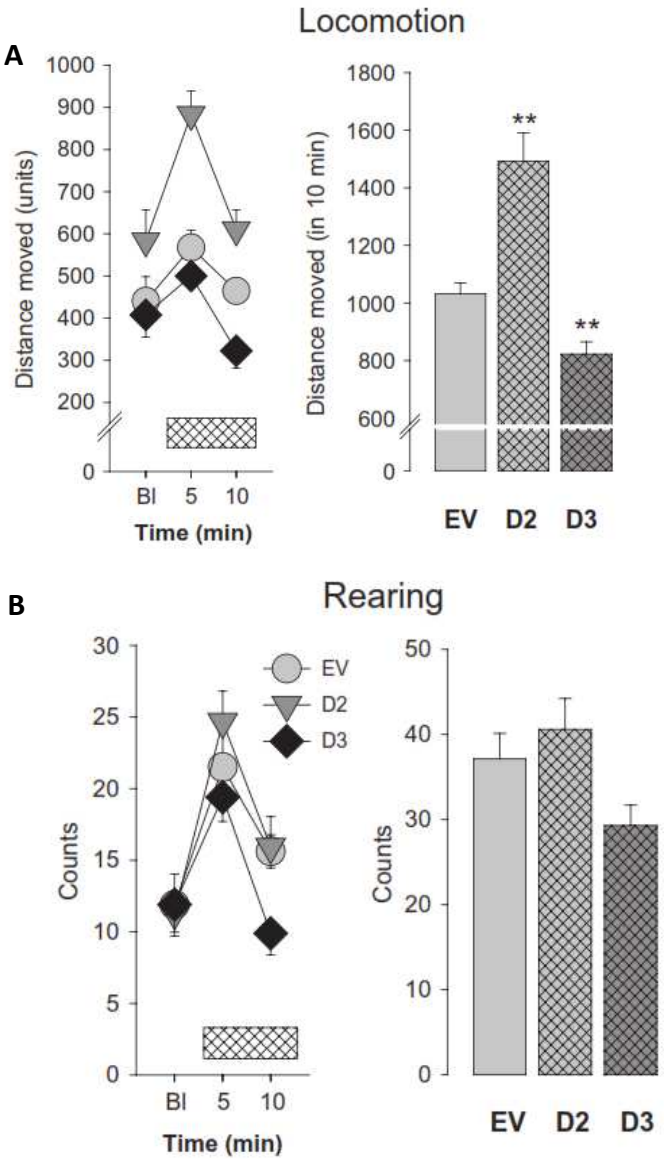


Figure 19: The effect of nucleus accumbens D2R and D3R knock down on light-induced locomotor activity (mean + S.E.M.).

*Left figures show the time course of the light-induced activation (hatched bar – time of light- stimulation; Bl - baseline. Bar graphs show genotype differences when activity was collapsed over 10 minutes of stimulation) *p>0.05, ** p>0.01, ANOVA followed by t-tests vs. Empty vector (EV) control.*

Left panels show that upon introduction of a light stimulus, overall locomotor (A) and rearing behaviours (B) were increased in all three groups including EV controls. A) Light induced locomotor behaviour was significantly increased in animals injected with D2R-expressing lentiviruses vs. EV (p=0.002) and, in contrast, reduced for D3R overexpression (p=0.002). B) Light-induced increase in rearing behaviour was not significantly affected by D2R or D3R receptor overexpression (p > 0.05) compared to EV.



3.4 Discussion

3.4.1 Summary of results

The results presented here aimed at investigating the specific contributions of D2R and D3R, expressed in the NAcc, in novelty- and light- induced locomotor activity. Lentiviral-mediated gene overexpression and targeted gene knockdown were used to specifically and locally manipulate expression of these genes. *In vitro* transduction in HEK-293T cells of these lentiviral vectors showed significant overexpression as well as efficient and specific knock down of these receptors. Bilateral stereotaxtic lentiviral injections were performed in the NAcc of rats and subsequently novelty and light induced locomotor activity were investigated. It was found that NAcc D2R and D3R share a similar function in spontaneous locomotor activity in a new environment though no contribution of these receptors to locomotor activity in a familiar environment was found. However, there was a difference in their role in visual stimulation-induced locomotor activity. NAcc D2R seems to limit light-induced activity, as silencing NAcc D2R potentiated this behaviour. On the other hand, NAcc D3R slightly reduced light-induced activity. In order to corroborate this data, *in vivo* validation of DA-receptor overexpression and knock down is required. Immunohistochemical staining of the brain sections from the different groups demonstrated that the injections were indeed localised in the NAcc and that the lentiviruses targeted specifically neuronal cells in this region.

3.4.2 Novelty - induced locomotor activity

The functional role of D2R and D3R in novelty-induced (spontaneous) locomotor activity has been extensively studied in receptor knockout mice and using pharmacological ligands. Because of the limitations associated with these methods, lentiviral - mediated gene delivery approach was applied, which enables specific spatio-temporal gene manipulation. The data presented here demonstrates that knockdown of NAcc D2R (both isoforms) resulted in a significant decrease in locomotor activity on day 1 of testing (representing a novel environment), but not on day 2, when habituation has occurred. Mice lacking D2R have been reported to display decreased locomotor activity (Baik *et al.*, 1995; Aoyama *et al.*, 2000; Fowler *et al.*, 2002), which is in agreement with the data presented here. Also consistent with our findings are studies using D2R antagonists, suggesting that decrease in D2R function has an inhibitory effect on spontaneous locomotion. For

example, chronic blockade of D2R using metoclopramide, a selective D2R antagonist led to a decrease in sponataneous locomotion in rats (Braun *et al.*, 1997). Other studies have also obtained similar findings using different D2R antagonists (Millan *et al.*, 2004; Huang *et al.*, 2010a). Administration of a D2R antagonist, sulpiride, resulted in a dose-dependent decrease in locomotor behaviour whereas quipirole, a D2R agonist dose-dependently increased locomotor behaviour (Stuchlik *et al.*, 2007). NAcc-specific D2R stimulation has been shown to increase locomotor activity in rats (Gong *et al.*, 1999). These studies, together with our findings, demonstrate that D2R has an inhibitory effect on spontaneous locomotor activity.

The data from this study also indicate that silencing of D3R in the NAcc resulted in decreased locomotion. This hypoactivity was generally less pronounced than that obtained by D2R knockdown and was only significant after the initial 5 minutes on the first day of testing. The behavioural consequences of D3R silencing presented here are in contrast to earlier reports. Previously, antisense oligonucleotides targeted against D3R (Ekman *et al.*, 1998) has found to increase locomotor activity while the idea that D3R plays an inhibitory role in locomotion is also supported by another study (Menalled *et al.*, 1999). Pharmacological studies report attenuating effects of the D3R preferential agonists, 7-Hydroxy-2-(di-n-propylamino) tetralin (7-OH-DPAT) and PD 128907 on locomotor activity in rodents (Gyertyan & Saghy, 2004; Millan *et al.*, 2004). Furthermore, these D3R agonists, when infused directly into the NAcc also led to a decrease in locomotor function (Ouagazzal & Creese, 2000; Millan *et al.*, 2004). On the other hand the hyperlocomotor effects of D3R antagonists are reported by a wide range of studies (Waters *et al.*, 1993a; Waters *et al.*, 1993b; Klingpetersen *et al.*, 1995; Sautel *et al.*, 1995; Clifford & Waddington, 1998; Manzanedo *et al.*, 1999; Carr *et al.*, 2002; Gyertyan & Saghy, 2004; Millan *et al.*, 2004; Pritchard *et al.*, 2007). As highlighted in these reports, a possible account for the contradictory results may be due to the lack of selectivity of these ligands (Burris *et al.*, 1995; McNamara *et al.*, 2006). Further support for this reasoning comes from the observation that three different D3R agonists decreased locomotor activity in wild type and D3R knockout mice to similar levels (Boulay *et al.*, 1999b).

Studies using D3R knockout mice reveal contrasting findings as D3R deficient mice were reported to display levels of spontaneous locomotor activity that are either at (Karasinska *et al.*, 2005; Chourbaji *et al.*, 2008), below (Boulay *et al.*, 1999b) or above (Accili *et al.*, 1996; Xu *et al.*, 1997) the level of wild type mice. Adaptive mechanisms occurring in the knockout mice (Jung *et al.*, 1999) may account the inconsistencies in the above results. For example, in the D3R knockout mice were

found to express increased dopamine transporter mRNA, have increased dopamine reuptake and decreased tyrosine hydroxylase in the striatum compared to wild type (Le Foll *et al.*, 2005). Moreover, rodent species have varied genetic backgrounds which hinder the interpretation of pharmacological treatments (Ralph & Caine, 2005), and may account for the above discrepancies. For instance, C57BL/6J mice display higher novelty-induced locomotion than DBA/2 mice which may be, due to C57BL/6J mice having lower D3R mRNA expression (McNamara *et al.*, 2006).

Interestingly, the reduced locomotor phenotype of D2R-/- mice has been found to be further exacerbated by deletion of D3R (Jung *et al.*, 1999), suggesting that D2R and D3R share a similar function in regulating spontaneous locomotion. The data presented in this study confirm and expand this hypothesis, showing that such shared functions occur in the NAcc. Further supporting our findings, is the recent discovery that D2R and D3R interact physically and functionally with one another (Scarselli *et al.*, 2001), forming D2R/D3R heterodimers that are preferentially activated by the anti-parkinsonian agents, S32504, pramipexole and ropinirole (Maggio *et al.*, 2003)

Although knockdown of NAcc D2R and D3R resulted in obvious phenotypes, enhancing expression of these receptors in the NAcc had little effect on novelty-induced behaviour. Previously, transgenic mice over-expressing D2R and GFP, display hyperlocomotor phenotype in a novel environment (Kramer et al. 2011). And, D2R stimulation increases locomotor activity (Ralph & Caine, 2005; Stuchlik *et al.*, 2007), also when specifically occurring in the NAcc (Gong *et al.*, 1999). One possible explanation for the lack of effect of the D2R and D3R overexpression seen in this data might be that both receptors are well expressed in the NAcc, already at saturating levels and may have been internalised for example. Alternatively, the exogenous receptors might have not efficiently integrated into functional networks *in vivo*.Carrying out a radioligand binding assay would allow to verify if there are increased functional receptors on the membrane surface. Although even if this is the case, in order to see a effect, perhaps additional dopamine may be required to bind to the increased receptors on the membrane surface. It should also be noted that while the shRNA used to knockdown D2R targeted both $D2R_S$ and $D2R_L$ isoforms, only the effects of $D2R_S$ overexpression was investigated. Distinct roles for the two D2R isoforms were suggested in selective knockout approaches (Usiello *et al.*, 2000; Wang *et al.*, 2000). Mice expressing $D2R_S$ but not $D2R_L$ display reduced levels of spontaneous locomotion (Wang *et al.*, 2000; Hranilovica *et al.*, 2008), implicating that $D2R_S$ may play an inhibitory role in controlling locomotion and/or $D2R_L$ has a stimulatory role. In fact, $D2R_L$ deficient mice which still express functional $D2R_S$, at the same level of D2R in wild type mice suggest that there is a compensatory increase in $D2R_S$ expression in the

mutants. It would be worthwhile to perform stereotaxic injections of NAcc $D2R_L$ expression as well as knockdown to delineate the effect of both isoforms on locomotor behaviour.

3.4.3 Light-induced activity

Light-induced activity provides a reliable method of generating a behavioural response to a novel discrete visual stimulus in rats in a familiar environment (Muller *et al.*, 2007a; Pum *et al.*, 2009b). Previously the method of on-off light stimulation has been found to induce an increase of horizontal locomotion and rearing behaviour in a stimulus-intensity dependent manner (Pum *et al.*, 2008). Within the low intensity range used in this study (<440 lux), the light stimulus is not aversive but triggers exploratory behaviour (Pum *et al.*, 2009b) and appears to be a novel stimulus with some incentive salience for the animal. This interpretation is in line with a number of studies showing that the on- and off-set of low intensity visual stimulation works as a reinforcer in rodents (Roberts *et al.*, 1958; Hughes, 2007; Olsen & Winder, 2009) as well as in primates (Blatter & Schultz, 2006).

Our results suggest that D2R and D3R in the NAcc have opposite roles in the processing of visual stimuli, with D2R inhibiting locomotor activity induced by a visual stimulus and D3R promoting this behaviour. The observed effects may result from the activation of DA neurons in the ventral tegmental area (Horvitz *et al.*, 1997). Indeed, it has been proposed that DA neurons transmit additional nonreward signals in response to surprising, novel, salient and even aversive experiences (Bromberg-Martin et al., 2010). Our results support and extend this notion, showing that activation of D2R and D3R in the NAcc has distinct effects on light-induced locomotor response. Other evidence for a role of the D2R in processing of visual stimuli is provided by (Doi *et al.*, 2006)), showed that D2R-/- mice had reduced light-induced hypoactivity, compared to their wild type counterparts. However, their experiment is not directly comparable to the data presented in this chapter, as they investigated the light-induced suppression of locomotor activity elicited by changes in light-dark cycles.

There is an interesting similarity between the light-induced exploratory activity experiments presented in this study and prepulse inhibition (PPI) of the acoustic startle response— an operational model of sensorimotor gating. PPI, observed as a reduction in the startle response following a weak stimulus or 'prepulse' preceding the startling stimulus, is disrupted by altered DA levels in the NAcc (Swerdlow *et al.*, 1990) and involvement for both D2R and D3R in PPI have been

well documented (Swerdlow *et al.*, 1992; Wan & Swerdlow, 1993; Swerdlow *et al.*, 1998; De Bruin *et al.*, 2001; Swerdlow *et al.*, 2009). Although not directly comparable, our results indicate contributions of both receptors in light-induced activity, which also reflects interaction between a sensory stimulus (in that case visual) and motor processing.

3.4.4 Further consideration, limitations and conclusion

The differences in locomotor activity observed in our experiments could have been explained by differences in anxiety levels between groups. To test this, the elevated plus-maze test (Walf & Frye, 2007) was used. No anxiety related measures were significantly affected in this test. Our results are not surprising despite a number of studies implicating (though often with contrasting results) D2R and D3R in anxiety-like behaviours (Steiner *et al.*, 1997; Drago *et al.*, 1998; Black *et al.*, 2002; de Oliveira *et al.*, 2006; Micale *et al.*, 2009). The amygdala, rather than the NAcc, is thought to modulate fear and anxiety through mechanisms possibly involving D2-like receptors (de la Mora *et al.*, 2010).

One consideration to take note of is the fact that, unlike the overexpression construct LeGO-iG2, the knockdown plasmids LeGO-G and LeGO-C, have separate promoters for the cloned insert and the reporter gene. This raises the question of the shRNA essentially being expressed when we only assessed for GFP expression. Therefore, in order to further improve the study design, *in vivo* validation of both overexpression and knockdown constructs are required. Although an a behavioural effect is seen, it will be useful to quantify the expression of both D2R and D3R *in vivo* either at mRNA or protein level in order to validate gene silencing and overexpression. Attempts have been made to achieve this using *in situ* hybridisation without any success as even endogenous D2R and D3R mRNA was not detected. In this case, the RNA probes were not used previously so further optimisation would be required in the probe design so that they hybridise efficienctly. Also, it is thought that the RNA may have degraded in the process. Furthermore, immunostaining using a number of D2R and D3R targeting antibodies which failed to detect any protein expression in the brain slices. Several concentrations of the primary and secondary antibodies were tested which did not work. Another technique that could be used is careful isolation of the NAcc tissue for qPCR analysis of mRNA levels of both genes as well as for protein analysis by Western Blot.

A further consideration regarding the spread of the lentiviral particles in the nucleus accumbens needs to be considered. Although the spread of lentiviral particles has been subjectively investigated by determining the spread by immunostaining for GFP. It would be useful to quantify the extent of spread of the lentivirus from the injection site. From my observations, the injection sites and lentiviral spread were generally consistent in each rat. For larger brain regions such as the striatum, some publications perform more than one stereotaxic injections to ensure that the whole region is covered. Nonetheless, quantifying the coverage of the lentiviral particles is important especially because some genes may have a subtle effect on behaviour and in order to see an effect, most neurons will need to be infection. Another consideration is the fact that only neurons have been infected despite the fact that the CMV promoter is non-specific in terms of driving transgene expression in different cell types although it has been shown to have variable expression in different human and rodent cell lines (Qin *et al.*, 2010). For this project, it is important to verify that the GFP expression is exclusively seen in neurons and not glia. Thus, in addition to GFAP, another glial-specific marker S100b, could have been used for immunohistochemical analysis to confirm that the transgenes are not expressed.

In conclusion, this study demonstrates that lentivirus-mediated gene delivery can provide important insights into the brain region-specific functionality of individual genes in behaviour. Using viral mediated gene delivery, long term and localised neuronal overexpression of, or selective knock down of, D2R and D3R in the NAcc were obtained. Our results suggest that while NAcc D2R and D3R are both required for novelty-induced locomotor activity, they are less important once an environment became familiar. Importantly, based on our knock down results, this data demonstrate opposite roles of NAcc D2R and D3R in locomotor activity elicited by a novel, discrete light stimulus in a familiar environment. Data from this chapter will contribute to understanding the differential functional roles of D2R and D3R in mediating behavioural responses. Furthermore, our results also demonstrate that this technique can be applied to study the behavioural consequence of novel candidate genes such as *Auts2* linked to a particular disorder.

Chapter 4

Cellular characterisation of *AUTS2*

4.1 Chapter Aims

As discussed in the general introduction chapter, despite its apparent implications in a number of neuropsychiatric disorders, the cellular and molecular function of *AUTS2* is largely unknown. The aim of this chapter is to:

1) Investigate its sub-cellular localisation, and

2) Gain insight into its biological function by identifying its interacting protein partners.

In order to achieve this, a plasmid expression vector overexpressing *AUTS2* either in its native form or as a fusion protein containing a V5 tag was designed. 293T cells were transiently transfected with these constructs and western blot was carried out to a) test a commercially available antibody targeting AUTS2; b) detect successful expression of AUTS2 at the protein level. Immunocytochemistry was also performed to determine the cellular localisation of AUTS2 in cells transfected with the overexpression constructs. In addition, attempts to identify its binding proteins by immunoprecipitation have been presented here.

4.2 Introduction

AUTS2 was initially identified as an autism susceptibility candidate gene in a pair of monozygotic twins (MZ) with autism who had identical balanced chromosomal translocation at t(7;20) (q11.2; p11.2) which disrupted the *AUTS2* gene (Sultana *et al.*, 2002). Other studies have also identified disruptions in *AUTS2* in patients with different severities of autism and/or mental retardation (MR) (Sultana *et al.*, 2002; Richler *et al.*, 2006; Kalscheuer *et al.*, 2007; Huang *et al.*, 2010b). As reviewed in Chapter 1, recent genetic findings implicate its involvement in a number of psychiatric disorders such as ADHD (Elia *et al.*, 2010), schizoaffective disorder (Hamshere *et al.*, 2009), epilepsy (Komoike *et al.*, 2010; Mefford *et al.*, 2010) and more recently, alcohol addiction (Schumann *et al.*, 2011; Zuo *et al.*, 2012).

Notably, comparison of the Neanderthal genome to that of modern day human revealed that *AUTS2* was one of many genes positively selected early in human development which, the authors suggest, may be due to its potential contribution in cognitive processes (Green *et al.*, 2010). Moreover, it has been cited that the amino acid sequence of the nuclear localisation sequence is highly conserved in human, mouse and chicken (Bedogni *et al.*, 2010b) providing further evidence for its biological importance as a possible nuclear protein.

4.2.1 *AUTS2* – molecular function

The human Autism Susceptibility Candidate 2, *AUTS2,* gene spans 1.2 Mb of genomic DNA and is located on Chromosome 7 (q11.22- q11.23); it is also referred to as *KIAA0442*. There are three validated transcript variants of this gene obtained as a result of alternative splicing which are translated into fairly large proteins as illustrated in Figure 20 and Table 17.

Figure 20: The different isoforms of human *AUTS2* on Genome Browser (Human Feb 2009 (GRCh37/hg19) Assembly).

Entrez gene ID: 26053 and Uniprot ID: Q5DO49

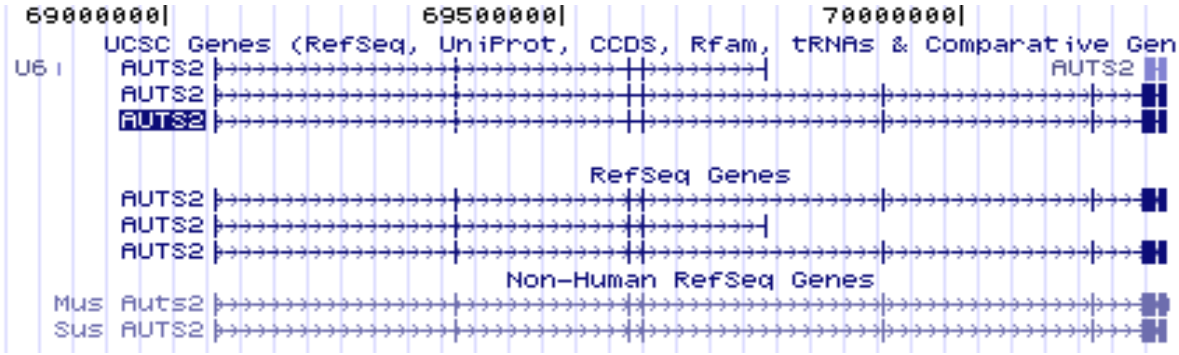


Table 17: Details of the three different isoforms of human *AUTS2*.

Protein molecular weight sizes were obtained from UniProt.

***AUTS2* (transcript variant)**	**ORF Size (bp)**	**NCBI mRNA ID**	**NCBI Protein ID (molecular weight in Daltons)**	**UCSC**
(1) Long	3780bp	NM_015570.2	NP_056385.1 (138,982)	uc003tvw.3
(2) Medium	3708bp	NM_001127231.1	NP_001120703.1 (136,416)	uc003tvx.3
(3) Short	800bp	NM_001127232.1	NP_001120704.1 (29,323)	uc003tvv.4

AUTS2 mRNA expression in the human brain tissue is present at high levels in the adult frontal cortex, caudate putamen and amygdala (Schumann *et al.*, 2011). However, its expression pattern is not brain specific. The human *AUTS2* mRNA is also strongly expressed in skeletal muscle and kidney with lower levels in the placenta, lung, and leukocytes (Sultana *et al.*, 2002) as well as in the liver (Hevner *et al.*, 2001; Schumann *et al.*, 2011). Analysis of porcine tissue mRNA showed high expression in muscle and brain and to a lesser extent ovary (Sato *et al.*, 2011).

However, the molecular and cellular role of *AUTS2* is largely unknown. The detection of highly conserved nuclear localisation sequences at the N-terminal of the *AUTS2* sequence suggest it may be a nuclear protein (Bedogni *et al.*, 2010b). Indeed, immunocytochemical staining of 293T cells overexpressing *AUTS2* showed that this protein was specifically localised to the nucleus. In the same study, immunohistochemical staining of mouse brain sections show that it is present specifically in neuronal nuclei (Bedogni *et al.*, 2010b). Nonetheless, its specific role is not yet elucidated and warrants further investigation.

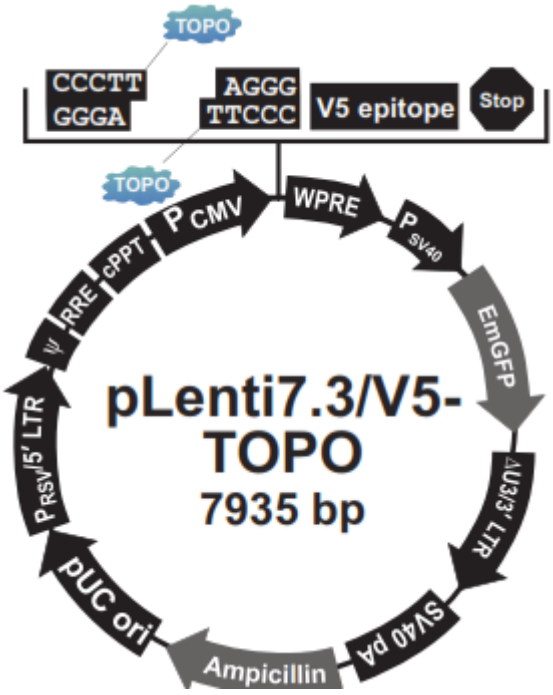
4.3 Methods and Materials

4.3.1.1 Construction of human *AUTS2* overexpression vectors

Two plasmid expression vectors were constructed that encoded *AUTS2* either in its native form or as a fusion protein with the V5 tag at the C-terminal. The pLenti7.3/ V5 – TOPO® vector backbone is shown in Figure 21.

Figure 21: The pLenti7.3/ V5 – TOPO vector.

This lentiviral expression plasmid contains a CMV promoter, a EmGFP reporter gene, an ampicillin resistance gene. A V5 epitope is present in the multiple cloning site which can result in a tagged protein of interest if cloned in frame with the V5 sequence.



Primer design for *AUTS2* amplification

Primers were designed to amplify the 3708bp (medium) isoform of human *AUTS2*. The forward primer incorporated the KOZAK consensus ([G/A]NNATGG**)** sequence – the optimal sequence required for translation initiation. Two reverse primers were designed to obtain either the AUTS2 protein sequence in frame with the V5 epitope tag resulting in a fusion protein (AUTS2

+ V5 or V5-tagged AUTS2) or the native AUTS2 protein. Table 18 shows the primer sequence used for amplification. To obtain the native form of AUTS2, a stop codon was included in the reverse primer. While to obtain V5-tagged AUTS2 protein, the stop codon was removed and the underlined nucleotides were added to keep in frame with the V5 epitope.

Table 18: Primers used to amplify the medium isoform of human *AUTS2* (3708bp) open-reading frame (ORF) for cloning into pLenti7.3/V5 TOPO® TA vector.

In order to generate two forms of AUTS2 protein products (i.e. native and V5-tagged AUTS2), two different reverse primers were used. Both primers have a similar sequence except for the primer resulting in the fusion protein has the stop codon removed and replaced with two cytosines to keep the AUTS2 sequence in frame with the V5 sequence.

Primer	**Sequence 5' > 3'**
Forward primer	ACCATGGATGGCCCGACGCGG
Reverse primer (native AUTS2)	TCGGGCCTCGATATCCTT
Reverse primer (AUTS2 + V5)	CCTCGGGCCTCGATATCCTTCAGCG

PCR Amplification of human *AUTS2*

After several trials, the optimal PCR reaction for *AUTS2* amplification contained a final concentration of 1x High Fidelity Phusion® buffer (Thermo Scientific, UK), 200µM of each dNTP (Life Technologies, UK), 0.5µM forward primer, 0.5µM reverse primer (either native or V5), 1U High Fidelity Phusion® DNA Polymerase (Thermo Scientific, UK), 3% DMSO, 10ng template DNA and dH_20 to a final volume of 50µl. The addition of DMSO inhibits the formation of secondary structures in the template DNA. The cycling conditions are shown in Table 19. The PCR reaction was run by electrophoresis on a 0.8% (w/v) agarose gel as detailed in 2.4.2 and the fragment band corresponding to the expected size of *AUTS2* (approximately 3.7kb in length) was isolated, gel-purified (as detailed in section 2.6.2.3) and stored at -20°C until further use.

Table 19: PCR thermal cycling conditions used for amplifying human *AUTS2*

Step	Time	Temperature (°C)
1 - Initial denaturation	30 seconds	98
2 - denaturation	10 seconds	98
3 - annealing	30 seconds	68
4 - extension	2 minutes	72
5 - number of cycles	Go to step 2 - 35 times	
6 – final extension	10 minutes	72

Addition of 3' A-Overhangs Post-Amplification

High Fidelity Phusion® DNA Polymerase lack the terminal transferase activity which adds the 3'A overhangs required for the ligation process. Therefore, following PCR amplification and fragment band purification, 3'A overhangs were added to the purified PCR product in a reaction containing a final concentration of 1x Taq buffer with magnesium, 1ng/µl PCR product, 1mM dATP (Life Technologies, UK) and 5U Taq Polymerase (Roche, Hertfordshire, UK) in a total volume of 10µl. The mix was heated at 72°C for 20 minutes and the ligation reaction below was carried out immediately.

Ligation of *AUTS2* into pLenti7.3/V5 TOPO expression vector

To perform the ligation, pLenti 7.3/V5 TOPO® TA cloning kit® (Life Technologies, UK) was used. The TOPO® cloning reaction consisted of: 1µl of the above PCR product with the 3' A overhang, 1µl salt solution, 1µl pLenti-TOPO® vector in a final volume of 6µl. In parallel, a control reaction was set up with no insert DNA to assess the re-ligation efficiency of the linearised vector. This mixture was incubated at RT for 20 minutes and then placed on ice. 3µl of this reaction was transformed into One Shot® Stbl2™ Competent *E.coli*.

Verification of correct DNA inserts

Following transformation, colony isolation and plasmid purification by minipreps; an enzyme digest was carried out to look for the presence of the insert in the vector and its orientation with respect to the vector. Therefore, additional digests were performed using restriction enzymes that would cleave once in the Multiple Cloning Site of the vector and also once in the *AUTS2* ORF at either the 5' or 3' end, not mid sequence. The BamHI enzyme met this criterion and thus was used to determine the orientation of the *AUTS2* ORF. A couple of miniprep DNA that contained *AUTS2* cDNA in the correct orientation were sent for sequencing to verify the sequence integrity of the insert using the primers listed in Table 20. Following correct sequence verification, maxipreps (section 2.6.2.1) were carried out for virus production. The above enzyme digest was repeated to confirm the presence of the insert before use.

Table 20: Primers used for sequencing of the human *AUTS2* open-reading frame (ORF) cloned into pLenti 7.3/V5 TOPO® TA vector

Primer	Orientation	Sequence 5' > 3'
AUTS2 seq1	Forward	CACCAAAATCAACGGGACTT
AUTS2 seq2	Forward	CGGGAAAGGCTCAGTGATAG
AUTS2 seq3	Forward	ATATGTTTGCCCCTCCCACT
AUTS2 seq4	Forward	GTGCTGCACACCCAACTG
AUTS2 seq5	Forward	GAGCCGGCCTACGAGAAC

4.3.1.2 Production of lentiviruses overexpressing *AUTS2*

The ViraPower™ Lentiviral Support Kit and Lipofectamine™ 2000 (Life Technologies, UK) was used for transfection according to the manufacturer's instructions for producing lentiviruses. The lentiviral packaging cell line was 293FT.

Briefly, the day before transfection, 5.5 x 10^6 293FT cells were plated (trypsinisation and cell count was done as per section 2.1.2.4 and 2.1.2.5) in 10cm sterile tissue culture dishes in a total volume of 10ml medium without antibiotics. On the day of transfection the culture medium was removed and replaced with 5ml Opti-MEM® I medium containing serum but no antibiotics. For each transfection sample, 9µg of the ViraPower™ Packaging Mix (Life Technologies, UK) and 3µg of the pLenti expression plasmid DNA) was added to 1.5ml Opti-MEM® I Medium without serum and mixed gently. Three plasmids were transfected: empty pLenti vector, pLenti + native *AUTS2* and pLenti + V5-tagged *AUTS2* . Separately, for each transfection reaction, 36µl of Lipofectamine™ 2000 was added to 1.5ml Opti-MEM® without serum and antibiotics and mixed gently. This mixture was incubated at RT for 5 minutes. The diluted Lipofectamine™ 2000 was subsequently added to the diluted plasmid DNA and viral packaging mix. This mixture was then incubated for 20 minutes at RT for the DNA-Lipofectamine™ 2000 complexes to form. Following incubation, the DNA-Lipofectamine™ 2000 complexes were added drop wise to each plate and mixed. The cells were then placed back in a humidified incubator at 37°C with 5% CO_2. The following day, the medium containing the DNA-Lipofectamine™ 2000 complexes was removed and replaced with 10ml complete culture medium without antibiotics and placed back in a humidified incubator at 37°C with 5% CO_2. The virus containing supernatants were harvested 24 and 48 hours later. These were centrifuged at 3000rpm for 15 minutes at RT to pellet the cell debris. Following centrifugation, the supernatants were filtered through 0.22µm pore nitrocellulose, aliquoted and stored at -80°C until use. Finally RNA and protein cell lysates were extracted (see sections 2.7 and 2.9).

4.3.1.3 Transduction of 293T cells with lentiviruses expressing *AUTS2*

293T cells were infected with lentiviruses overexpressing either native or V5-tagged *AUTS2* as well as the empty vector as detailed in section 2.3.2.2. Lysates were collected for RNA and protein extraction as detailed in sections 2.7 and 2.9 respectively. However, only approximately 2% transduction efficiency was achieved using this protocol. Having optimised the protocol several times, by increasing viral particles, longer incubation times with particles, there was no improvement in infection efficiency. This may have been to low titre obtained

during transfection since the transfection efficiency observed based on the number of GFP positive cells was high (~80%-90%) 48 hours post transfection.

4.3.1.4 Transient transfection of 293T cells with plasmid DNA

As mentioned above, the lentiviruses produced using the above protocol was inefficient in transducing 293T cells. Transient plasmid DNA transfection was carried out in 293T cells. This was performed in 6 well plates as per 2.2.2.1. Transfection was done for the below constructs and carried out in triplicates for RNA extraction, protein extraction and for immunostaining: 1) no plasmid DNA - only transfection mix; 2) empty vector; 3) overexpression - native *AUTS2*; 4) overexpression - *AUTS2* + V5.

4.3.1.5 qPCR and analysis

The primers used to amplify exogenous *AUTS2* mRNA were designed to detect the medium isoform (3708bp) acknowledged by both RefSeq and the UCSC genome browser. Human *ACTIN* was used as the housekeeping gene against which expression values for *AUTS2* were normalized to produce ΔCt values ($\Delta Ct = Ct_{AUTS2} - Ct_{ACTIN}$). Ct was defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. The ΔCt value for each condition was subtracted from the ΔCt of the control condition i.e. cells transduced with empty vector. The changes in mRNA expression relative to this control was obtained by raising 2 to the negative power of the calculated difference i.e., for *AUTS2* overexpression: $2^{-(\Delta ct\ AUTS2\text{-}overexpression\ -\Delta Ct\ empty\ vector)}$. The primers sequences are given in Table 21.

Table 21: Primers used to amplify the AUTS2 and β -ACTIN for qPCR

Primer	Sequence 5' > 3'
AUTS2 medium isoform (forward)	GCCATTGTCAGCCTACAACA
AUTS2 medium isoform (reverse)	GTGGTGGGAGATGTGAGGTG
β-ACTIN (forward)	GCTCGTCGTCGACAACGGCTC
β-ACTIN (reverse)	CAAACATGATCTGGGTCATCTTCTC

4.3.1.6 Western Blot

Protein extraction and western blot was carried out as described in 2.9, using the antibodies and dilutions shown in Table 22.

Table 22: Primary and secondary antibodies, dilutions and blocking buffer used for western blot.

TBST is 1x Tris Buffered Saline with 0.025% Tween

Blocking buffer	Primary antibody	Dilution		Secondary antibody	Dilution
TBST+ 5% milk	Anti-V5	1: 5000		Odyssey anti-mouse 800CW	1:10000
TBST+ 5% milk	Anti- AUTS2	1:1000		Odyssey anti-rabbit 680	1:5000
TBST+ 5% BSA	Anti –ACTIN	1:1000		Odyssey anti-rabbit 680	1:5000

4.3.1.7 Immunocytochemistry

Immunocytochemistry was carried out as described in section 2.9.2.1. The blocking buffer used was 10% goat serum in PBST containing 0.3M glycine. Primary and secondary antibodies and their dilutions are shown in the table below.

Table 23: Dilutions of primary and secondary antibodies used for immunoytochemistry.

Source and host species of antibodies are detailed in section 2.9.1.3.

Primary antibody	Dilution		Secondary antibody	Dilution
Anti-V5	1: 200		Alexa Fluor 594	1:500
Anti- AUTS2	1:200		Alexa Fluor 488	1:500

4.3.1.8 Co-Immunoprecipitation (Co-IP)

Co-IP of AUTS2 using the anti-AUTS2 and anti-V5 antibody was carried out as detailed in section 2.9.2.6.

4.3.2 Results

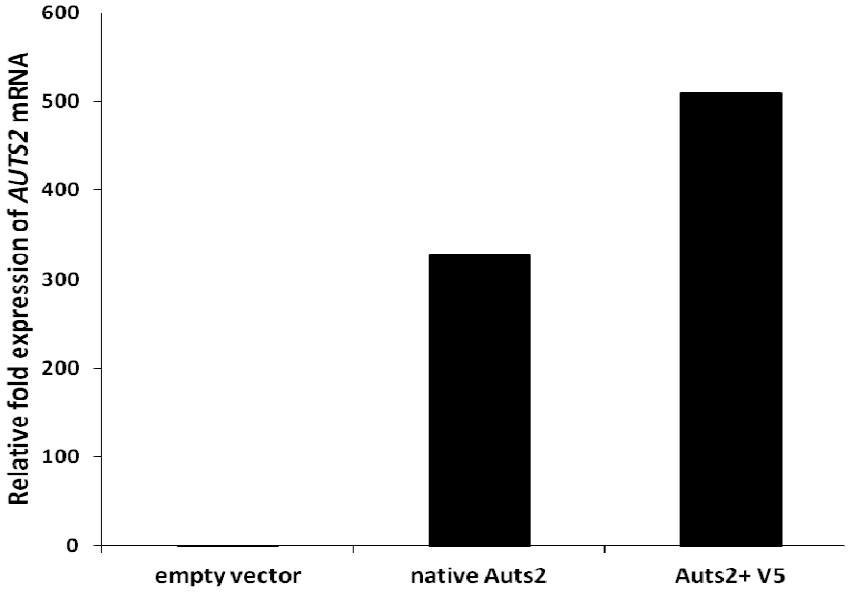
4.3.2.1 Subcellular localisation of AUTS2

Overexpression of AUTS2 and assessment of a commercially available anti-AUTS2 primary antibody

In order to determine its sub-cellular localisation, two lentiviral expression plasmids were generated that encoded either human *AUTS2* in its native form or as V5-tagged *AUTS2*. 293T cells were transiently transfected by calcium phosphate co-precipitation with these plasmids and empty vector; a transfection efficiency of approximately 75% was achieved. As seen fromFigure 22, qPCR analysis show that *AUTS2* mRNA expression is induced approximately 320-fold and almost 500-fold in 293T cells transduced with the lentiviral plasmid vector overexpressing native *AUTS2* and V5-tagged *AUTS2* respectively compared to expression with the empty vector. In parallel, in order to eliminate the fact that the induction of *AUTS2* mRNA expression was due to amplification of plasmid DNA contaminants in total RNA samples, as opposed to the corresponding cDNA, a reverse transcription reaction was set-up in parallel whereby the reverse transcriptase enzyme was not included, preventing cDNA synthesis. Thus any signal obtained from the amplification reaction would reflect only plasmid DNA contamination. Very low levels of expression were detected in this control reaction – Ct values for '*AUTS2* native' and '*AUTS2* + V5' were 31.2 and 29.4 respectively compared to their respective Ct values with the reverse transcriptase enzyme added (14.6 and 13.9 respectively), indicating that some contaminating plasmid was present in the cDNA samples, however, this accounted for a very small level of expression. Note: low Ct levels indicate high expression and vice versa.

Figure 22: *AUTS2* mRNA expression in 293T cells transfected with either empty vector or vector overexpressing *AUTS2*.

Values are analysed relative to empty vector control, and taking into account expression level attributable to contaminating plasmid DNA in the cDNA sample. AUTS2 mRNA expression is induced significantly in cells transfected with plasmid overexpressing either native AUTS2 or V5-tagged AUTS2 compared to empty vector. This data is representative of one experiment of two that were conducted; as a result error bars are not shown.

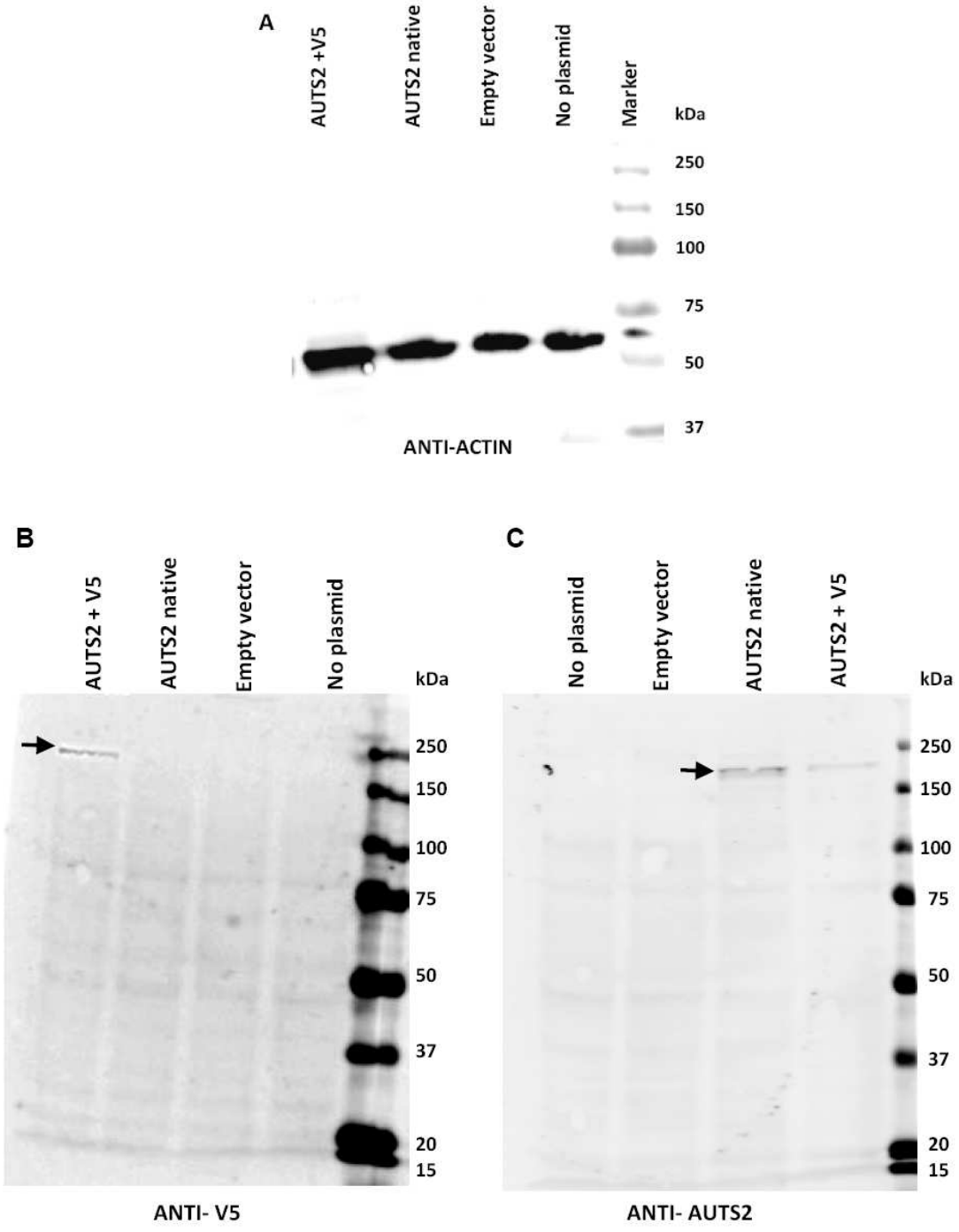


Having verified that *AUTS2* is induced at the mRNA level, establishing that it is also expressed at the protein level was required. This was done using two antibodies (anti- V5 and anti-AUTS2) for Western Blot analysis on protein extracts of 293T transfected with plasmids overexpressing human AUTS2 either in its native form or as a V5-tagged fusion protein, empty vector and no plasmid vector. Equal loading of the protein extracts was demonstrated in the control blots for which the anti-ACTIN antibody was used (Figure 23A). Using the anti-V5 antibody, which has been successfully used previously (Berghs *et al.*, 2001; Malagelada *et al.*, 2006; Irobi *et al.*, 2011; Ramonet *et al.*, 2012), revealed a band of a relatively higher than expected size of ~200kDa (Figure 23B) compared to the expected size of ~130kDa for human AUTS2, which was only present in the extracts overexpressing V5-tagged AUTS2 but not in other extracts, as expected. This demonstrated that V5-tagged AUTS2 over-expression is achieved successfully at the protein level and the higher than expected band size could be explained by another protein still bound to AUTS2. Another possible reason is that this could be another uncharacterised isoform of AUTS2 which is of a higher molecular weight.

Also, a commercially available antibody targeting AUTS2 from Abcam, UK, which has not been previously tested, was assessed here. The abovementioned antibody was predicted to recognise human AUTS2 according to the manufacturers' guidelines and was thus required to be tested prior to application in protein methods used subsequently in this thesis, such immunocytochemistry and immunoprecipitation. The AUTS2 antibody was tested by Western blot on protein extracts from 293T cell lines transfected with the same constructs as above. Figure 23C, shows that using this antibody, AUTS2 was indeed overexpressed at the protein level as a band of approximately 200kDa was present in the wells containing protein extracts from cells overexpressing native AUTS2 and V5-tagged AUTS2 but not in the empty vector or no plasmid extracts indicating three things: 1) that this anti- AUTS2 does indeed recognise human AUTS2 protein; 2) AUTS2 is successfully overexpressed at the protein level in its native form 3) that endogenous AUTS2 is not present at detectable levels in this cell line using this antibody. It must be noted that although overexpressed, the band detecting AUTS2 using both antibodies is faint despite loading a large amount of protein extract (30µg protein extracts) as shown by a very thick band for the control, ACTIN (Figure 23A). Owing to the higher than expected band size, in order to verify that the band corresponds to AUTS2 and/or another protein bound to it, identification by mass spectroscopy is required.

Figure 23: Western blot to determine the level of exogenous AUTS2 expression in 293T cell line transiently transfected with plasmids overexpressing *AUTS2*.

A) β-Actin control (molecular weight ~45kDa) is detected in all extracts. B) The anti-V5 antibody detects a specific band (indicated by arrow) of ~200kDa only in cells transfected with plasmid overexpressing V5 tagged Auts2 (AUTS2+V5). C) A band of the same size (indicated by arrow) was also detected by the anti-AUTS2 antibody (Abcam, UK) in cells overexpressing both native AUTS2 and V5-tagged AUTS2. Expected molecular weight of human AUTS2 protein is 130 ~kDa.



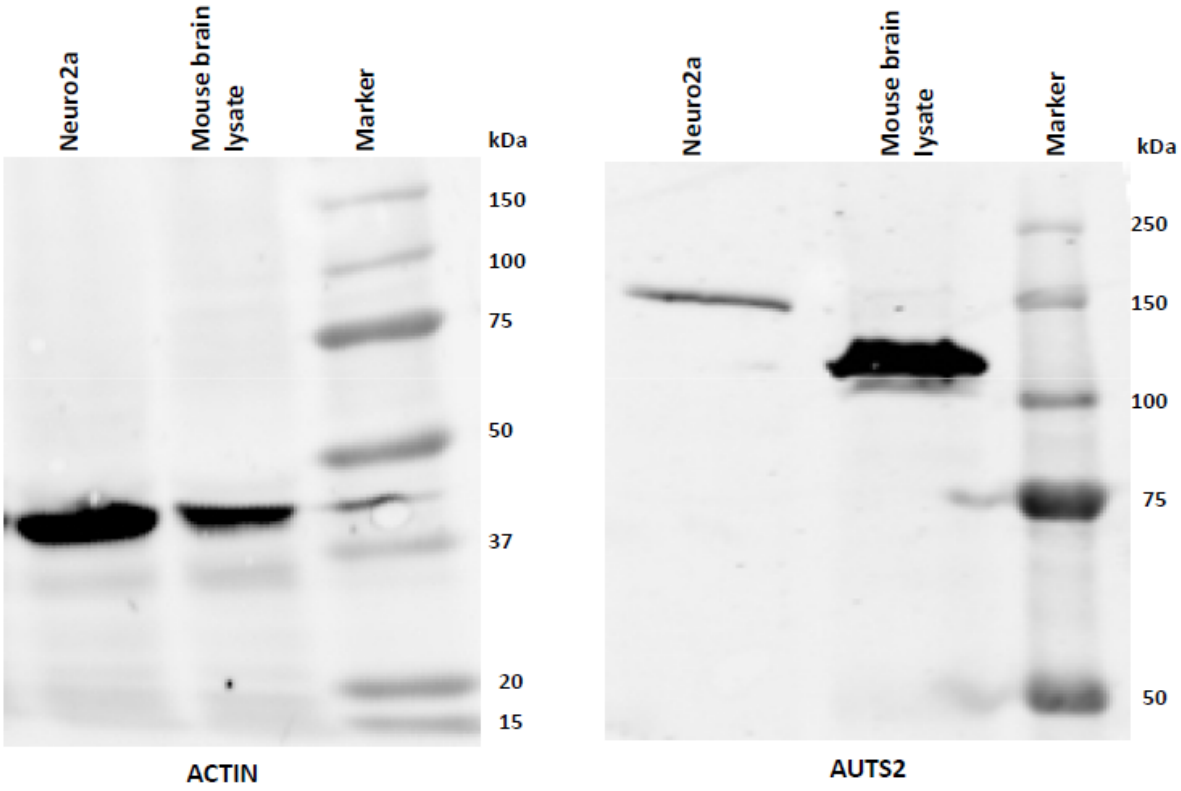
AUTS2 is expressed in mouse whole brain extracts and Neuro2a cell line

Western blots were carried out on a mouse neuroblastoma cell line, Neuro2a, and mouse whole brain to verify if this antibody recognises the mouse AUTS2 protein. The antibody epitope target sequence is present in mouse AUTS2 protein. Figure 24 shows a relatively intense band of an expected size of ~140kDa detected in the well containing mouse brain lysates. However, a band of a higher than expected size (~150kDa) was identified in the mouse Neuro2a lysates (Figure 24); This could indicate a different isoform of mouse AUTS2 which may be exclusively expressed in the Neuro2a cell line. Alternatively it could indicate that there may be another small protein still attached AUTS2 and thus appearing at a higher than expected size. In order to identify if the band indeed corresponds to AUTS2 and/or another protein, mass spectrometry for example liquid chromatography tandem mass spectrometry (LC/MS/MS) could be performed.

Another observation is that there is considerably more protein in the mouse brain lysate than in the cell line, this could be explained by the fact that there is higher expression of this protein in the brain than the cell line.

Figure 24: SDS-PAGE analysis to determine the level of AUTS2 protein expression in whole mouse brain lysate and Neuro2a (mouse neuroblastoma) cell line.

The anti-β-Actin antibody successfully detected β-Actin in both lysates with a molecular weight of ~45 kDa as expected. The AUTS2 antibody (Abcam) also detected a clear specific band in both cells although they were slightly different sizes: ~150 kDa in the mouse Neuro2a cell lysate and an expected size of ~140 kDa in the mouse brain lysate. 30µg of protein extract was loaded.



AUTS2 is localised in the nucleus

Having ascertained that the plasmid expression vector successfully induces *AUTS2* expression at the protein level and the antibody specifically recognises both human and mouse AUTS2, immunocytochemistry was performed to determine cellular localisation of AUTS2 in 293T and Neuro2a cell lines using either the anti-V5 and anti- AUTS2 antibodies.

293T cell line was first transfected with plasmid V5-tagged AUTS2 and as seen in Figure 25, although higher transfection efficiency (approximately 70%) was achieved, a lower number of GFP+ve cells are seen due to the cells being re-seeded onto specialised culture chamber slides for immunostaining. Nonetheless, in this way, staining from non-transfected cells can be compared to that of transfected cells in the same field of view. Deducable from the results,

firstly is co-localisation of the Green Florescent Protein marker (expression from the plasmid n vector), with the V5 antibody staining, shows that AUTS2 was overexpressed in these cells (Figure 25 a-c). In addition, co-localisation of V5 and DAPI (a nuclear counter stain) shows that AUTS2 is localised within the nucleus of 293T cell line (Figure 25c-e). Endogenous AUTS2 in the Neuro2a cell line was also localised in the nucleus using the anti-AUTS2 antibody (Figure 26 a-c).

Figure 25: Nuclear sub-cellular localisation of AUTS2.

a-e) 293T cell line were transfected with the plasmid vector overexpressing V5-tagged AUTS2 as shown by the GFP flourescence from the lentiviral vector (green) and anti-V5 antibody staining (red). Merged images of GFP and V5 are colocalised as indicated by arrows. Merged images of V5 and DAPI staining (blue) and V5 positive cells indicate that AUTS2 is localised in the nucleus (designated by arrows). Bar represents 50µm.

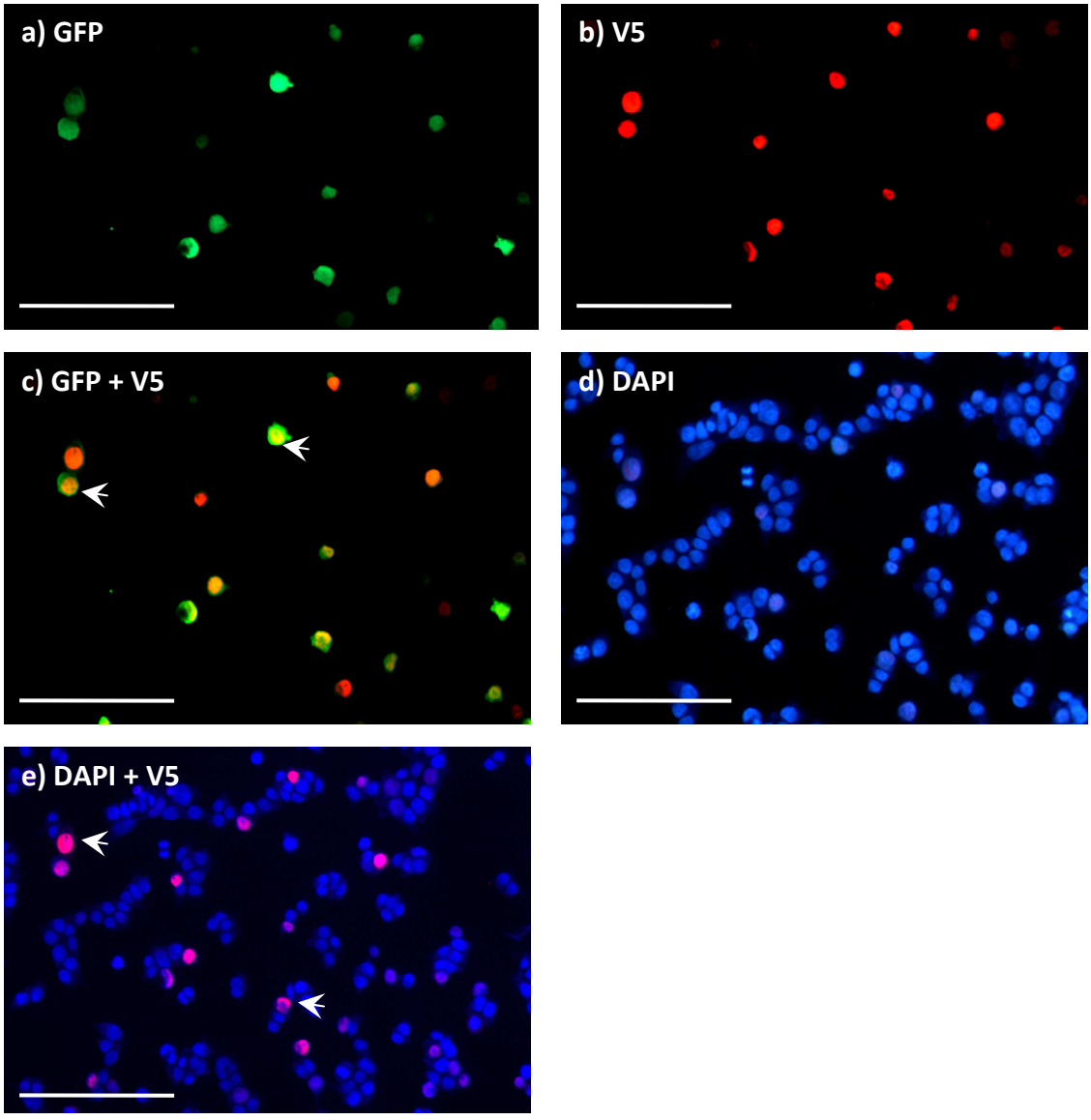
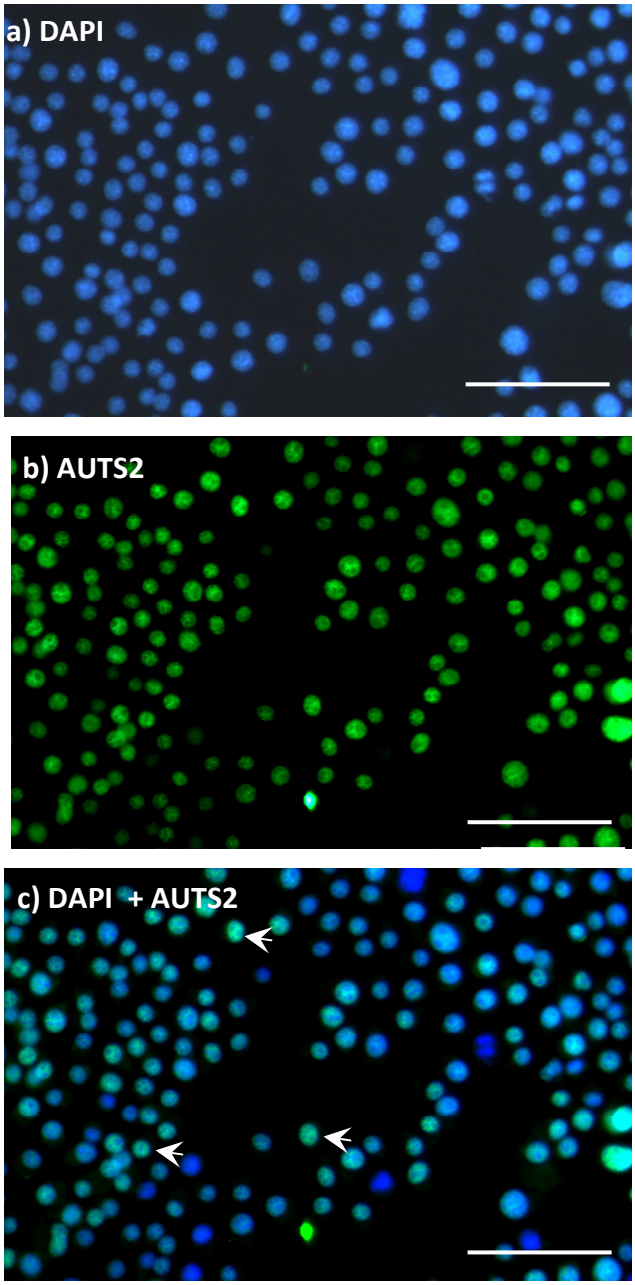


Figure 26 : Nuclear sub-cellular localisation of AUTS2.

a-c) The anti-AUTS2 was used to determine subcellular localisation of endogenous AUTS2 in Neuro2a mouse cell line. Positive endogenous AUTS2 staining is shown in green. Merged images (teal colour) of DAPI staining (blue) and anti-AUTS2 antibody staining (green) indicate that AUTS2 is localised in the nucleus (indicated by arrows). Images are taken at 40x magnification. Bar represents 50 µm.



4.3.2.2 Potential interacting partners of AUTS2

Co-Immunoprecipitation (Co-IP) of AUTS2

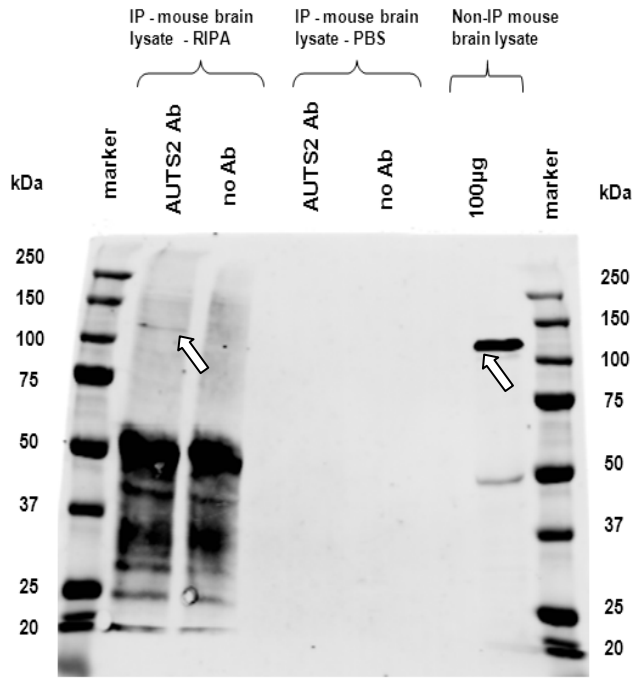
In order to find out its protein- protein interactions, co-IP of AUTS2 was performed with the aim to 'pull out' any interacting partners that would point out to its role in the nucleus. The anti-V5 and anti-AUTS2 antibody were assessed for their suitability for immunoprecipitation. Despite several attempts to optimise this protocol, the anti-AUTS2 antibody failed to specifically immunoprecipitate AUTS2 and its interacting partners in mouse brain lysate. As seen from Figure 27, A a very faint band of the expected size of AUTS2 was recognised by this antibody along with a substantial amount of non-specific binding. A smear of bands similar to the well with mouse brain lysate were present in the control (no primary antibody). Attempts to reduce this background by washing in PBS to have a more stringent condition led to no band detection at all.

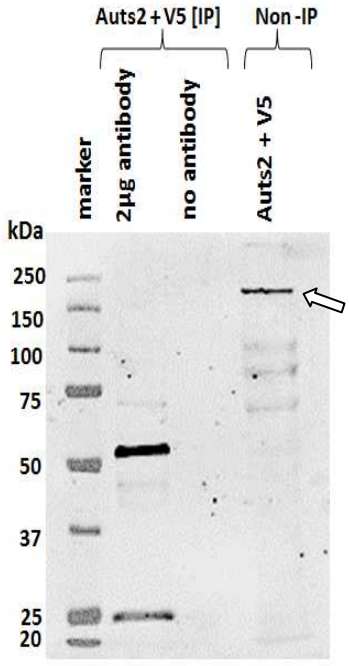
On the other hand, using 293T cells overexpressing AUTS2 in the human 293T cell line, the anti-V5 antibody did not pull out V5-tagged AUTS2 as no band was detected corresponding to its expected size (Figure 27,B). Two other bands were visible which were equivalent to the size of the heavy and light chain for this antibody. The lack of immunoprecipiated AUTS2 could be explained by the need for a higher transfection efficiency; in this experiment a transfection efficiency of approximately 60% was achieved. Also as AUTS2 may be localised in the nucleus as shown above, nuclear protein extracts would enrich AUTS2 which may enable to 'pull out' AUTS2 more efficiently.

Figure 27: Western blot to assess the efficacy of the A) anti-AUTS2 antibody and B) anti-V5 antibody for immunoprecipitation (IP) of AUTS2 protein.

A) 500 µg mouse whole brain lysate was used to purify AUTS2 protein by IP using the anti-AUTS2 antibody. A Western blot was performed to ascertain whether AUTS2 had been purified successfully. The anti-AUTS2 antibody is not effective for purification of AUTS2 in mouse brain lysate as a substantial amount of background is detected despite a faint band comparable to that detected in the non-purified lysate is visible (indicated by arrows). B) 100µg 293T cell lysate overexpressing V5-tagged AUTS2 was purified using the anti-V5 antibody. A band comparable to that detected in non-immunoprecipitated sample (indicated by arrow) was not detected. Instead the bands that were detected are those corresponding to the light and heavy chain of the anti-V5 antibody. 'No antibody' represents the negative control; i.e., the absence of antibody; Ab = antibody.

A **Mouse brain lysate** **B** **293T human cell line**





4.3.2.3 Summary and discussion

The purpose of this chapter was to determine the sub-cellular localisation of AUTS2 and identify its potential interacting protein partners in order to gain some insight into its function. In order to ascertain its sub-cellular localisation, firstly, two lentiviral expression plasmids were generated; one which encoded human *AUTS2* in its native form and another that overexpressed a small epitope tag, V5, C-terminally fused to *AUTS2* (denoted V5-tagged *AUTS2*). 293T cells were transiently transfected with these plasmids and, in parallel, with an empty vector as control. RNA and protein extracts were obtained so as to 1) verify the induction efficiency of the plasmid overexpression constructs at the mRNA and protein level and 2) evaluate the specificity of a commercially available anti-AUTS2 antibody.

Quantitative PCR analysis demonstrated a high *AUTS2* mRNA induction. Its expression was also induced at the protein level, evident from a Western blot analysis of 293T cells transfected with V5-tagged AUTS2 using the anti-V5 antibody. Additionally, the anti-AUTS2 antibody was assessed for its target specificity in the same Western blot analysis that also contained lysates overexpressing AUTS2 in its native form, which successfully demonstrated that it recognises AUTS2. However, in the abovementioned blots, a higher than expected molecular weight band was detected by both antibodies. This may be due to post-translational modifications such as phosphorylation, myristoylation and glycosylation; putative sites for these modifications have been reported previously (Sultana *et al.*, 2002). Additional reference to Uniprot indicated that AUTS2 is a phosphoprotein (uniprot code: Q8WXX7). Ultimately, data from the above findings demonstrate that anti-AUTS2 and anti-V5 antibodies specifically recognise their target protein.

Secondly, to determine the subcellular localisation of AUTS2, fluorescent immunostaining was performed in 293T cells overexpressing the V5-tagged form of this protein. Co-localisation of this protein (stained for by the anti-V5 antibody) with the counter-stain DAPI confirmed that AUTS2 is a nuclear protein. This finding may explain the faint band obtained in the abovementioned SDS-PAGE analysis for both antibodies. Nuclear rather than whole cell protein extracts may enrich the amount of AUTS2 protein obtained in order to see the high levels of expression. A consideration here is the fact that a human cell line that endogenously expresses AUTS2 would be appropriate to use. Although, to consolidate the above findings, SDS-PAGE analysis of extracts from a mouse neuroblastoma cell line Neuro2a was performed

to detect endogenous AUTS2 expression at the protein level using the anti-AUTS2 antibody assessed above. As with the results above, a higher than expected molecular weight size was detected, potentially indicating post translational modifications. To determine its sub-cellular localisation, immunostaining in Neuro2a showed co-localisation of endogenous AUTS2 within the nucleus (DAPI counterstain) confirming that AUTS2 is a nuclear protein in this cell line. Together, these results are in agreement with a previous report which demonstrated its localisation in neuronal nuclei in the mouse brain (Bedogni *et al.*, 2010b).

Next, having determined its cellular localisation, it was necessary to find out what exact role it played in the nucleus. Co-immunoprecipitation (Co-IP) was attempted using the validated anti-AUTS2 and anti-V5 antibodies with the aim of investigating its function by identifying its interacting protein partners. Co-IP was performed to pull-down the AUTS2 protein from cultured cells transiently overexpressing the native form of AUTS2 or V5-tagged AUTS2 along with other proteins either bound to it directly or as part of a protein complex. Initial assessment intended to determine suitability of the anti-AUTS2 antibody for immunoprecipitation using mouse brain lysate showed that it may have captured a small amount of AUTS2 but a high degree of background was detected in all samples including control (no primary antibody included in the lysate). Despite several protocol optimisations, such as longer pre-clearing time and using increased protein lysates, the above results either persisted or no band was visible at all indicating that this antibody is not be suitable for immunoprecipitation. Trial with the anti-V5 antibody for 'pulling out' AUTS2 and its associated proteins, lead to no visible band. A possible explanation for the lack of success in this technique may be that AUTS2 expression is not at an adequate level for pulling out. As the previous immunostaining data suggest that it is a nuclear protein, this may be ameliorated by enriching its purification by performing nuclear protein extracts as opposed to whole cell extracts, carried out here. Two other commercially available antibodies have previously been tested in our group, which were also found to be unsuitable for immunoprecipitation.

In order to gain some indication of proteins that interact with AUTS2, references to several publically available databases containing information on protein structure, function and interactions or those that can predict this, were interrogated with one database showing interactions of AUTS2 between a number of putative proteins, many of which are involved in neurobiological and neurodevelopmental processes. Various bioinformatic resources that provide annotation for known and predicted PPIs were interrogated to ascertain whether

there is currently any evidence of AUTS2 interacting protein partners. STRING 9.0 software revealed a number of predicted PPIs (Figure 28), though these data are based on findings from text mining; there is no actual experimental evidence of any physical interaction between any of these proteins and AUTS2. Nonetheless it is interesting to note that some of these supposed interacting proteins play a role in developmental neural circuitry as described in Table 24. The remaining bioinformatic resources had no record of PPIs involving AUTS2. Nonetheless as demonstrated recently, tandem affinity purification (TAP) of 293TREx cells stably expressing Polycomb group ring fingers PCGF3 and PCGF5, recovered AUTS2, FBRS and FBRSL (Gao *et al.*, 2012). These highly conserved proteins are part of a chromatin modifying multi-component complex called PRC1-like complex involved in the regulation of embryonic development through transcriptional repression of as yet unknown genes. As TAP enables the identification of stable PPIs, it is possible that AUTS2 may be part of or interacts with this complex in order to mediate developmental effects. Further investigation in this direction is clearly required.

Figure 28: *In silico* analysis of potential protein-protein interactions (PPIs) involving human AUTS2 protein.

Output from STRING 9.0 software illustrating putative PPIs based on evidence from text-mining. The nodes represent individual gene products whilst the lines indicate interaction between relevant genes.

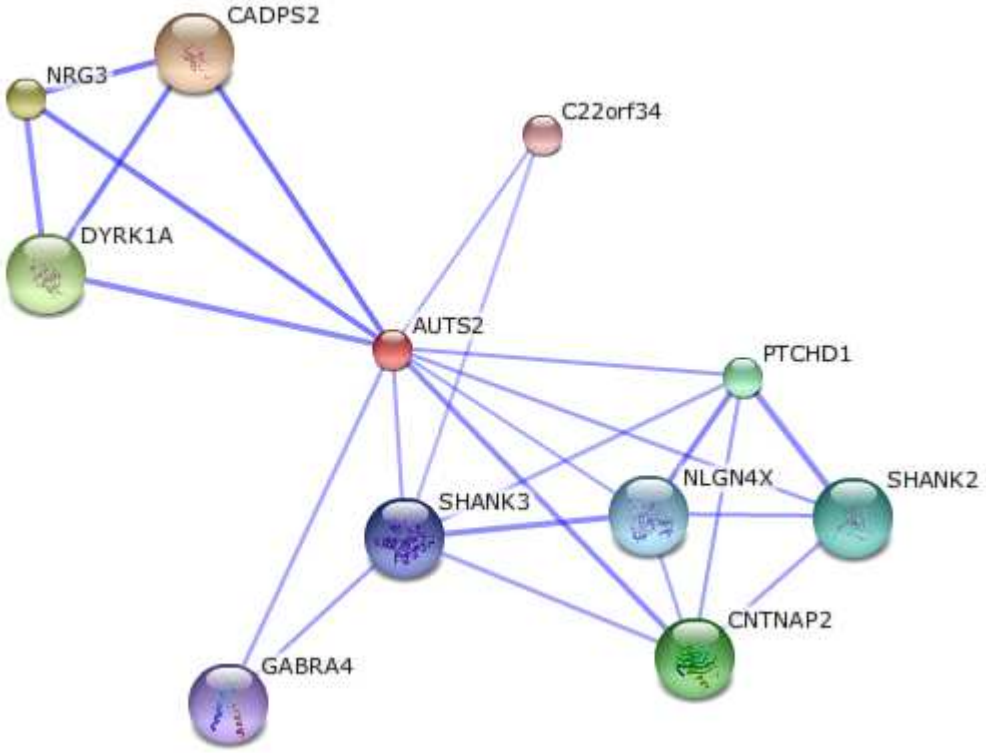


Table 24: Function of putative AUTS2 interacting protein partners obtained from STRING.

Potential interacting Protein	Summary of function	Effect on / association with AUTS2	References
TBR1 *T-box brain gene 1*	A transcriptional regulator involved in brain development.	Tbr1 is thought to regulate AUTS2 expression (Green *et al.*, 2010).	(Bedogni *et al.*, 2010a)
CADPS2 *Calcium dependent secretion activator*	Regulates the exocytosis of vesicles from neurons and neuroendocrine cells.	Both AUTS2 and CADPS2 have been strongly associated with autism and were both positively selected during human evolution (Green *et al.*, 2010).	(Speidel *et al.*, 2003)
DYRK1A *Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A*	Thought to play a role in signalling pathways regulating cell proliferation and may be involved in brain development.	Both AUTS2 and DYRK1A have been strongly associated with autism and were both positively selected during human evolution (Green *et al.*, 2010).	(Tejedor & Hammerle, 2011)
NRG3 *Neuregulin 3*	Involved in neuroblast growth, migration and differentiation among other cell types.	Both AUTS2 and NRG3 have been strongly associated with autism and were both positively selected during human evolution (Green *et al.*, 2010).	(Zhang *et al.*, 1997)
CDH9 *Cadherin 9*	Cell-cell adhesion protein that functions early in development and is thought to play a role in neural development.	Tbr1 knockout mice displayed reduced levels of AUTS2 and CDH9 (Bedogni *et al.*, 2010a).	(Williams *et al.*, 2011)
CNTN4 Contactin 4	Disruptions in this neural cell adhesion protein have shown to be involved in autism and brain development	Chromosomal inversion disrupting AUTS2 and CNTN4 in children with Autism spectrum disorders (Bakkaloglu *et al.*, 2008).	(Hansford *et al.*, 2003).

A possible approach that could be used to find out about PPIs of AUTS2 is the yeast-two hybrid system. Very briefly, this would involve cloning *AUTS2* into a bait vector which also contains another gene encoding a DNA-binding domain from a transcription factor (e.g. Lex A), thus generating a Bait-DNA-binding domain fusion protein. On another plasmid vector, a putative interacting protein (e.g. PCGF5) designated the term 'prey' is cloned in frame to an activation domain of the Gal14 yeast transcription factor. If the two proteins (bait and prey) physically interact, this would bring the DNA-binding domain of one vector and the activation domain in the other vector in close proximity to restore a functional transcription factor. Thus the reporter gene (e.g. LacZ) is transcribed resulting in the blue coloration of the yeast cells which would otherwise not occur. This can be repeated for any number of prey proteins and would verify previous findings as mentioned above and determine novel interacting partner proteins.

In conclusion, this sub-chapter has successfully constructed plasmid overexpressing AUTS2 in its native form as well as V5-tagged AUTS2. These constructs were used to validate a commercially available anti-AUTS2 antibody which successfully detected its target. Previously, other commercially available antibodies assessed in our lab, failed to detect AUTS2. This chapter has thus identified an antibody that specifically targeted AUTS2. Using the validated antibody and overexpression constructs, it was demonstrated that AUTS2 was localised in the nucleus in two cell lines from different species (human and mouse). Although co-immunoprecipitation failed to reveal its interacting protein partners and the above *in silico* analyses have served to provide clues concerning the potential functioning of AUTS2 protein, *in vitro* research is clearly needed to try to verify and extend upon these findings. Given its association to a number of neuropsychiatric disorders, the next chapter aims to investigate its role in neurodevelopment.

Chapter 5

Lentiviral mediated gene silencing of *AUTS2* in human foetal neural stem cells, SPC04

5.1 Project Aims

Given that *AUTS2* is a neurodevelopmental gene implicated in a number of neuropsychiatric disorders, this chapter aims to expand our understanding of its function in brain development using a human neural stem cell line to model neurodevelopment *in vitro*. Figure 29 illustrates a brief outline of the intended plan for this part of the thesis.

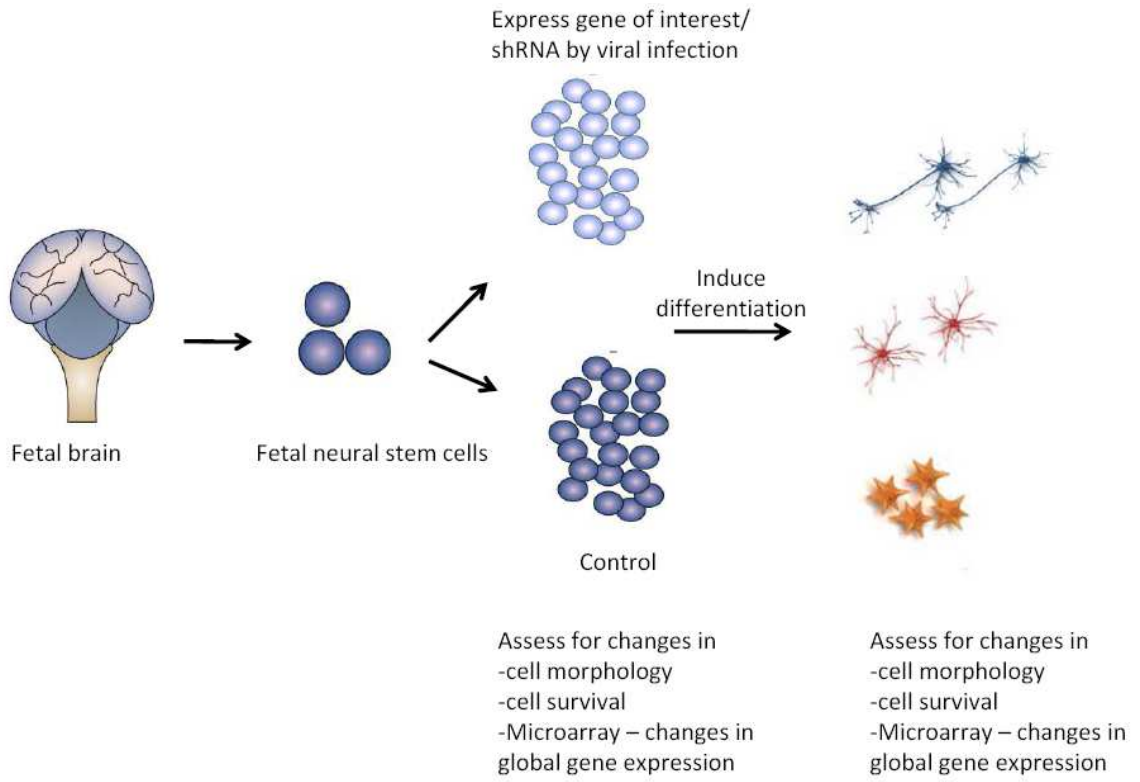
More specifically, the aims of this chapter are to:

1) Ascertain the role of *AUTS2* in neurogenesis and neural differentiation, key process for neurodevelopment.

2) Investigate changes in gene expression patterns of other genes in response to *AUTS2* knockdown upon differentiation of neural stem cells.

Figure 29: A brief overview of the methods used to investigate the functional role of AUTS2 using human foetal neural stem cell line, SPC-04.

Adapted from (Jakel et al., 2004). The overall research plan is to transduce SPC-04 in order to silence AUTS2 expression and assess for changes in cellular features such as cell survival, cell proliferation, and differentiation at the undifferentiated stage as well as various stages of differentiation. As analysed by RT-PCR, AUTS2 expression is high and consistently maintained at this level in various stages of differentiation in this cell line (unpublished data). In addition, a global gene expression profile will be carried out using microarrays to look at differential gene expression of those involved in neurodevelopment in particular.



5.2 Introduction

5.2.1 Neural stem cells (NSCs) as an *in vitro* model to study neurodevelopment

In addition to animal studies, the use of neural stem cells has contributed substantially to research in the molecular mechanisms underlying neurodevelopment and how genetic as well as 'environmental' alterations can impact this process. With the view that most psychiatric and neurodegenerative disorders may be due to aberrant processes taking place during development, currently a major focus of research is directed towards studies using stem cell lines.

Stem cells are undifferentiated cells that have the ability to self renew through cell division and have the potential to differentiate into multiple cell types also known as multipotency. Stem cells can be derived from embryonic, foetal as well as adult tissues. Human embryonic stem cells are derived from the inner cell mass (group of cells that will develop into the embryo) of the embryonic blastocyst which can be induced to differentiate into any cell type. In culture, these cells form embryoid bodies (aggregates of differentiated and undifferentiated cells) which in turn can differentiate into the three primordial germ layers: ectoderm, mesoderm and endoderm. Under specific conditions, the embryoid bodies can be induced to differentiate into mature neurons and glia through an intermediate differentiation stage involving neural precursors (Reubinoff *et al.*, 2001). The latter can give rise to neurally derived cells. Neural development can be divided into three main stages: neural induction, neural specification, and neural maturation. Each of the stages in the developing embryo can be modelled *in vitro* in hESCs. They provide a useful tool in studying the various differentiation stages in neural development and understanding the mechanisms involved in brain development (Vaccarino *et al.*, 2011). NSCs can also be isolated directly from the foetal brain between 8 and 20 weeks' gestation (Carpenter *et al.*, 1999; Vescovi *et al.*, 1999) and can be expanded into large numbers in culture. The generation of specific cell types from these cells and their fates during development is regulated by factors that affect cell division, differentiation and apoptosis. Adult human neural stem cells can also be obtained and are increasingly being used in research as there are no ethical concerns relating to their use. However, they are difficult to expand in culture due to senescence after a few passages.

In mammalian development, neural stem cells (NSCs) appear during the period of neural induction, which occur after the differentiation of the germ layer from the epiblast. They have the ability to self-renew as well as generate all the different neural cell types (Carpenter *et al.*, 1999; Vescovi *et al.*, 1999). Fibroblast growth factor-2 (FGF2) and epidermal growth factor (EGF) along with insulin like growth factor enable the proliferative self renewal of NSCs (Arsenijevic *et al.*, 2001). To identify progenitor cells, most commonly, nestin, an intermediate filament, Mushashi, an RNA-binding protein, as well as Sox2, a transcription factor essential for maintaining pluripotency used as a markers for undifferentiated cells. While in the absence of mitogens, these cells can differentiate into neurons and glia (Svendsen *et al.*, 1998). Temporally, *in utero,* neurogenesis precedes gliogenesis (Shimazaki, 2003). Neurons and glial cells can be identified by their morphology and expression of cell-specific markers such as, for neurons, MAP2 (microtubule-associated protein 2) and βIII-tubulin (microtubule protein exclusively found in neurons) and glial cell specific markers like GFAP (glial fibrillary acidic protein) and S100b (glial-specific calcium binding protein B.

Brain development entails differentiation of specialised cell types and their organisation in precise special anatomy. As such, research into neurodevelopment and the underlying mechanisms involved will provide insight into the biological bases for neurodevelopmental and psychiatric disease. Genetic modification of stem cells is important for the study of gene function in early human development. Although gene transfer using transfection and electroporation methods have been employed (Eiges *et al.*, 2001; Furuya *et al.*, 2003; Zwaka & Thomson, 2003; Urbach *et al.*, 2004; Vallier *et al.*, 2004); they have either resulted in inefficient transfer (Eiges *et al.*, 2001; Zwaka & Thomson, 2003), or cytotoxicity (Furuya *et al.*, 2003; Zwaka & Thomson, 2003) or produce transient gene expression (Lakshmipathy *et al.*, 2004; Lorenz *et al.*, 2004). Increasingly, the use of viruses in genetic modulation of particularly neurodevelopmental genes in rodent and human stem cells has proven useful. Stable transduction of human stem cells using lentiviral vectors has been widely reported (Gropp *et al.*, 2003; Ma *et al.*, 2003; Kosaka *et al.*, 2004; Ben-Dor *et al.*, 2006; Clements *et al.*, 2006). Foetal neural stem cells (NSCs) have been shown to be successfully transduced with the viruses as vectors for gene transfer (Fu *et al.*, 2008). An example of its use, is lentiviral-mediated overexpression of the mutant *HD* in human NSCs derived from the striatum to investigate its effect on neural cell death upon differentiation and maturation (Tabrizi et al., unpublished data). Silencing of *DISC-1* in neural progenitor cells showed reduced neural migration supporting its vital role in neurodevelopment (Kobayashi *et al.*, 2010). There is a plethora of

such proof of principle findings in the literature demonstrating that neural stem cells are indeed a good model for studying neurodevelopment. As with all models there are disadvantages, being an in vitro model system, it lacks the entire contents of the brain functional system. Also, the cells in culture may be in a state of 'stress' due to handling and repeat passaging which may effect cellular and molecular mechanisms. Furthermore, the brain is constantly learning, experiencing different emotion, thoughts and feeling which cannot be modelled *in vitro*. Nonetheless it is important to obtain some indications and ideas on how neurodevelopmental processes occur to further our understanding of brain function.

5.3 Methods and Materials

5.3.1 Lentiviral constructs for *AUTS2* silencing

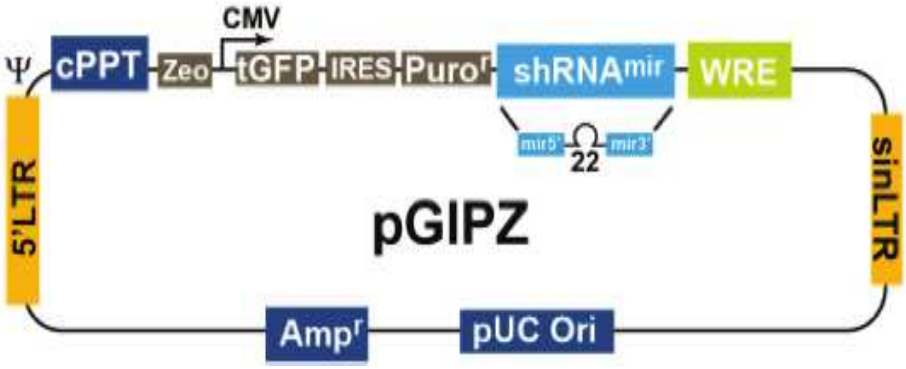
Lentiviruses were produced for the below plasmid constructs, all of which were obtained from Open Biosystems®, Thermo Scientific, UK. The pGIPZ lentiviral plasmid contains a CMV promoter, a puromycin resistance gene and TurboGFP marker among other common lentiviral features (Figure 30, A). The sense sequences for the siRNA (micro-RNA adapted shRNA, Figure 30,B) used are given below:

1) pGIPZ + non-silencing siRNA; sense sequence: ATCTCGCTTGGGCGAGAGTAAG
2) pGIPZ + siRNA_ *AUTS2* # 1; *AUTS2* target sequence: CTAAAGCTGTGTTCCCATA
3) pGIPZ + siRNA_ *AUTS2* # 2: *AUTS2* target sequence: CAGCACCTTGGGAAGAGAA

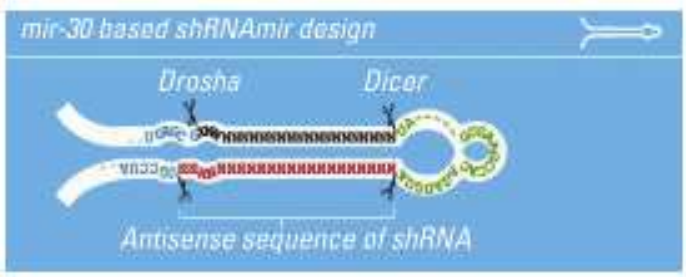
Figure 30: Features of A) pGIPZ lentiviral vector and B) siRNA design.

A) A commercially available plasmid (pGIPZ) cloned with the siRNA was obtained from Open Biosystems, UK. The CMV promoter is responsible for the expression of the reporter gene, turboGFP. This is followed by an encephalomyocarditis virus internal ribosome entry site (IRES), to facilitate the expression of the mammalian selection puromycin resistance gene and the siRNA. Other features include, cPPT: central polypurine tract to help translocation into the nucleus of non-dividing cells; WRE: Woodchuck response element to enhance the stability and translation of transcripts; SIN-LTR (Self Inactivating Long Terminal Repeats); pUC Ori to maintain high copy replication and maintancence of plasmid in E.Coli. and ampicillin resistance gene.B) the siRNA design is based on the primary microRNA-30 (mir-30) transcript.

A



B



5.3.2 Lentiviral production and ultracentrifugation

Open Biosystems® TransLenti Viral Packaging System (Thermo Scientific, UK) was used for transfection and production of lentiviral particles according to the manufacturer's instructions. Briefly, the day before transfection, 5.5x 10^6 293T cells were plated per 10cm dish in complete medium. For each transfection, 9µg transfer vector and 28.5µg packaging mix was diluted in 1ml serum free medium and separately, 187.5µl of Arrest-In was diluted in 1ml of serum free medium. The diluted DNA and the diluted Arrest-in reagent were combined and incubated at RT for 20 minutes before adding 3ml complete medium. Following this step, the growth medium was aspirated from the cells and the transfection complexes mix. The cells were placed back at 37°C in 5% CO_2 humidified incubator. Five hours later, the transfection mixture was replaced with 12ml culture medium and replaced in the incubator. Lentivirus-containing supernatants were harvested 48h and 72h following transfection. These were centrifuged at 3000 rpm for 20 minutes at 4°C to pellet cell debris, filtered in a 0.22µm pore nitrocellulose, aliquoted and stored at -80°C until use. The supernatant were ultracentrifuged as described in 2.3.2.3. General titres obtained using this protocol ranged from 1.0-1.2 x10^9 TU/ml.

5.3.3 Lentiviral transduction of 293T and SPC-04 cell lines

293T cells were transduced as described in 2.4. Successfully transduced cells were selected by addition of complete medium with 10µg/ml puromycin 48h after transduction.

SPC-04 cell culture and lentiviral transduction is comprehensively explained in sections 2.2 and 2.4. Puromycin was not used for positively transduced selection as the SPC-04 cell line is sensitive to this antibiotic nonetheless high transduction efficiency is achieved using the protocol described in 2.4. Transduction/ transfection efficiency was determined as described in Chapter 2, section 2.2.2.2.

5.3.4 qPCR analysis

RNA extraction and reverse transcription were carried out as per details in section 2.8. Quantitative RT-PCR was performed as in section 2.9. Human Actin was used as the housekeeping gene for qPCR analysis of cDNA produced from 293T RNA extracts while the control gene used for analysis of SPC-04 cDNA was *RPL18*, a gene that encodes one of the L18E family of ribosomal proteins that comprise the 60S subunit; its expression was found to be

relatively consistent at the different stages of neural stem cell differentiation. The primer sequences for human, *ACTIN*, *AUTS2* and *RPL18* are as follows:

Table 25: qPCR primers used to amplify *AUTS2*, *ACTIN* and the control gene *RPL18* in SPC-04.

The AUTS2 primer amplified the medium and long isoform.

Primer	Sequence 5' > 3'
Human *ACTIN* (forward)	GCTCGTCGTCGACAACGGCTC
Human *ACTIN* (reverse)	CAAACATGATCTGGGTCATCTTCTC
Human *AUTS2* (forward)	ACCCCATCCGGGACCCCTTG
Human *AUTS2* (reverse)	GGAGCCGCGTGTGCTCGTAG
Human *RPL18* (forward)	GAGAGGTGTACCGGCATTTC
Human *RPL18* (reverse)	CTCTGGCACGCTCGAACT

The primers used to amplify exogenous *AUTS2* mRNA were designed to amplify the long (transcript variant 1) and medium (transcript variant 2) isoforms acknowledged by both RefSeq and the UCSC genome browser as the shRNA targeted these isoforms. For data analysis, expression values for *AUTS2* were initially normalized against the internal control mRNA expression value to give ΔCt values ($\Delta Ct = Ct_{target} - Ct_{control}$). Ct was defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. The ΔCt value for each condition was subtracted from the ΔCt of the control condition i.e. cells transduced with non-silencing siRNA. The changes in mRNA expression relative to this control was obtained by raising 2 to the negative power of the calculated difference i.e. for siRNA_*AUTS2* ($2^{-(\Delta ct\ siRNA\ AUTS2\ infected\ cells - \Delta Ct\ non-silecning\ infected\ cells)}$).

5.3.5 Microscopy and imaging

Images were taken with the Lieca fluorescence microscope DM2500 with images taken at 10x and 20x.

5.3.6 RNA quantification, labelling and Microarrays

The RNA quantification, labelling, hybridisation, washing and scanning steps were carried out by Eric Nasser, Biomedical Research Centre (BRC) genomics facility, King's College London. Total RNA was quantified using the Qubit® RNA assay kit (Life Technologies, UK) on a Qubit®2.0 fluorometer according to the manufacturer's instructions. RNA integrity was checked using the Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer. Total RNA was labelled (biotinylation) using TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip® (Cambio, Cambridge, UK) according to the manufacturer's instruction, with RNA quantification using the Qubit® RNA assay kit on a Qubit®2.0 fluorometer as previously mentioned. Gene expression profiling was performed using the HumanHT -12 v4.0 expression BeadChip Kit (Illumina Inc, CA, USA) which contains more than 47,000 probes derived from the National Centre for Biotechnology Information Reference Sequence (NCBI). Arrays were read on an Illumina Bead array confocal scanner. Each sample was done in triplicates (n=3).

5.3.7 Statistics

qPCR quantification of *AUTS2* expression: Data were expressed as mean ± SEM and normalised to the values obtained from cells infected with non-silencing siRNA expressing lentiviruses. To verify a between treatment difference, a one-way ANOVA with factor treatment was performed. To test for treatment differences, pre-planned comparisons were conducted using the independent sample *t*-test.

Microarrays: Analysis was performed by Dr Venu Pullabhatla, NIHR GSTFT/KCL Comprehensive Biomedical Research Centre, using GenomeStudio Data Analysis Software with the following parameters: 1) Background subtraction; 2) Quantile normalization; 3) *t*-test model for identifying differentially expressed probes and genes; 4) Benjamini and Hochberg error correction for multiple testing (FDR).

For identifying differentially expressed genes, a Differential Score of +/- 13, is the equivalent of a P value <=0.05. +/- indicates directionality of the differential expression (up or down regulation).

5.4 Results

5.4.1 Silencing of *AUTS2* in 293T cell line

Two commercially available lentiviral plasmids encoding *AUTS2*-targeting siRNA were tested for efficient knock down in the 293T cell line which endogenously express all three isoforms of *AUTS2*. As 293T cells expressed *AUTS2* at a similar level to SPC-04 (Figure 31), this cell line was used to screen for siRNA that efficiently knockdown *AUTS2* as it easy to culture and less costly to maintain. Although a human neuroblastoma cell line SHSY5Y, was available in our lab, which would be well –suited for this prupose, its, AUTS2 expression level was low in this cell line compared to 293T cell line (Figure 31); hence the latter was used for the experiment.

293T cell line was transduced with lentiviruses containing these constructs alongside a control i.e. lentiviruses encoding non-silencing siRNA. As shown inFigure 32, cells transduced with lentiviruses expressing siRNA_*AUTS2* # 1 efficiently silences this gene down to approximately 30%. There was significant effect between groups on *AUTS2* expression ($F_{4,10} = 31.64$, $p<0.001$). Post-hoc analysis showed significant knockdown of *AUTS2* by this siRNA ($p<0.001$). Data is shown for the expression of the long and medium isoform, as this siRNA targeted these specifically. On the other hand, siRNA_*AUTS2* # 2 had no significant effect on *AUTS2* expression ($p=0.186$). Data is shown for the medium and long isoform although this siRNA targeted all three isoforms. Analysis of the expression of all three isoforms showed similar results as that shown below (i.e. no change in expression; data not shown).

Figure 31: RT-qPCR to confirm endogenous AUTS2 mRNA expression in three human cell lines.

Lower delta Ct values ($Ct_{tAUTS2} - Ct_{ACTIN}$) indicate higher levels of expression and vice versa. These findings indicate that AUTS2 mRNA is endogenously expressed in the three cell lines examined, with the highest expression levels evident in human 293T cell lines.

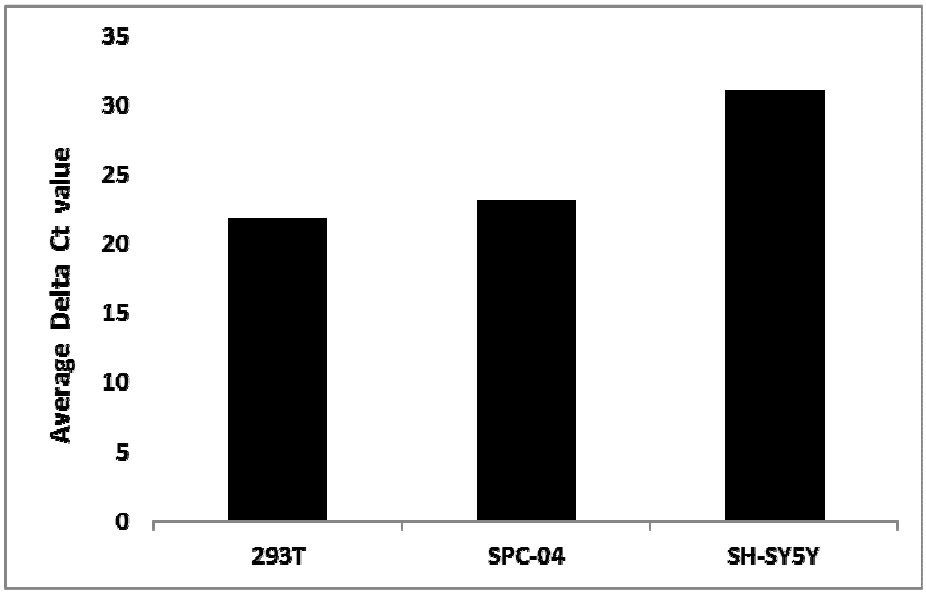
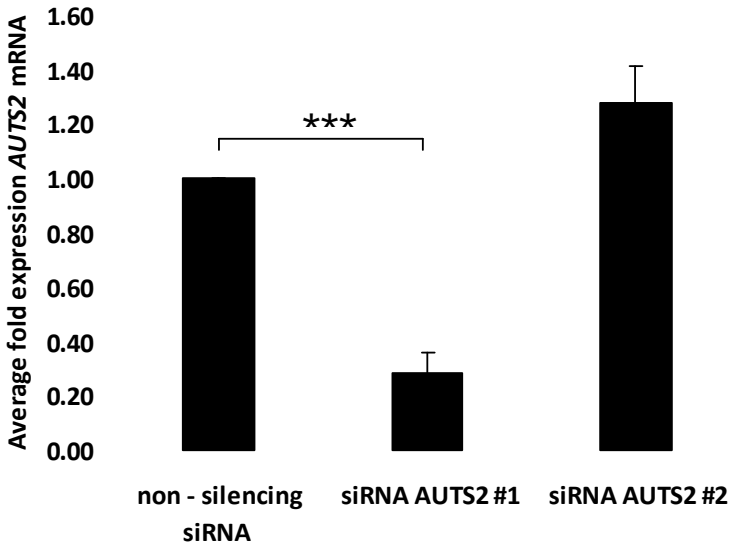


Figure 32: Effects of the *AUTS2*-targeting lentiviral constructs on *AUTS2* expression *in vitro*.

*HEK293T cells were infected with lentiviruses carrying a vector either encoding a sequence that does not target any mammalian sequence (denoted non-silencing siRNA) or encoding siRNAs targeting AUTS2. Quantification of mRNA levels was performed by real-time qPCR (mean ± S.E.M of 3 independent experiments), ***p<0.001, ANOVA followed by t-test vs non-silencing siRNA.*



5.4.2 SPC-04 cell line differentiation upon lentiviral mediated silencing of *AUTS2*

SPC-04 have the ability to generate a high number of neurons as described in (Jeffries *et al.*, 2012), where undifferentiated cells (Figure 33, A) after 7 days differentiation, acquire a more elongated morphology (Figure 33, B). This change is more pronounced after 14 days differentiation.

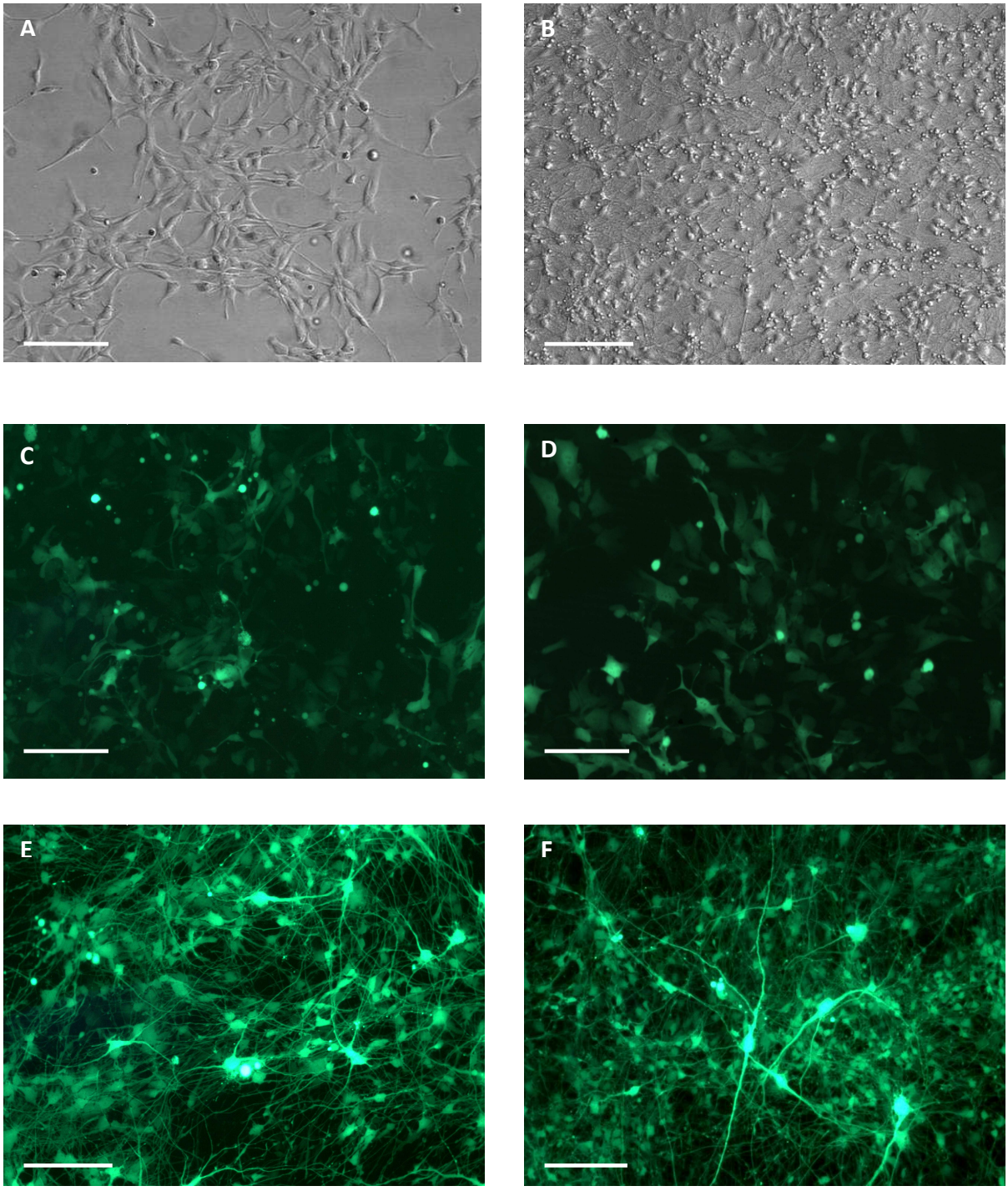
Having ascertained that siRNA#1 efficiently knocked down *AUTS2* in the 293T cell line, SPC-04 were transduced at the undifferentiated state after which lysates for RNA extraction were collected 48hours later. In addition, this cell line was also transduced during differentiation (i.e. addition of the lentiviral particles along with the differentiation-inducing agents). Lysates for RNA extraction were collected 7 days after the removel of the lentivirus and differentiation-inducing media (denoted 7 days differentiation). Refer to section 2.1.2.3 for

more protocol details on the various differentiation stages. A transduction efficiency of 85-90% was achieved in both these experiments.

Before harvesting the cells for qPCR and microarray analysis, the morphological features of the cell line were compared between cells infected with lentiviruses containing non-silencing siRNA and siRNA targeting *AUTS2* at the undifferentiated stage and after 7 days differentiation. Results indicated that there were no visible differences in morphology of the undifferentiated and differentiated cells between the two groups (Figure 33, C-D). The same applied to cells transduced during differentiation; there were no morphological differences between the two groups (Figure 33, E-F) at 7 days differentiation.

Figure 33: Representative images of SPC-O4 morphology before and after differentiation.

A) undifferentiated B) after 7 days differentiation C) Undifferentiated transduced with non-silencing siRNA D) undifferentiated AUTS2 siRNA E) after 7 days differentiation non-silencing siRNA F) after 7 days differentiation AUTS2 siRNA. Bar represents 200µm. GFP is the reporter gene expressed from the same CMV promoter regulating the siRNA expression.



5.4.3 Silencing of *AUTS2* in undifferentiated and differentiated SPC-04 cell line

Having verified that siRNA_*AUTS2* #1 efficiently knocked down its target, the human foetal neural stem cell line SPC-04 was transduced with this siRNA. As controls, cells were infected with lentiviruses encoding non-silencing siRNA and non-infected cells were included. Microarrays were performed in triplicates for 2 experiments: Experiment 1: cells transduced at the undifferentiated stage. Experiment 2: cells transduced during differentiation; lentiviruses were introduced during the addition of the differentiation inducing agents. Cells were harvested at various stages of differentiation i.e. pre-differentiation (after 48 hours incubation with medium containing differentiation agents and lentiviruses), 3 days after this stage denoted 3 days differentiation, 7 days differentiation and 14 days differentiation. Samples for 14 days differentiation were not included in the microarray due to low RNA yield and quality during RNA extraction. Disappointingly, microarray results showed no differential expression of any genes between any of the groups analysed including no knockdown of *AUTS2* detected (Table 26). No gene was identified with a Differential Score of greater than +13 or less than -13 in any of the comparisons performed indicating that no differentially expressed genes were identified. A Differential score of +/- 13 is equivalent to a *p* value < or = 0.05. The transfection efficiency was observed to be approximately 80% so lack of knockdown of *AUTS2* could not be attributed to lack of transduction. The entire dataset is not shown; however, Table 26 shows the microarray analysis for selected genes involved in neurodevelopmental processes for comparison between non-silencing and siRNA_*AUTS2* at 7 days differentiation. Noticeably, the standard deviation between arrays is larger than the mean which is one reason why no significant differential gene expression is observed in the entire dataset.

qPCR were also performed to confirm the above finding.

Experiment 1: Figure 34A, shows that cells transduced with lentiviruses expressing siRNA_*AUTS2* # 1 did not silence the gene in this cell line. There was no significant effect between groups on *AUTS2* expression ($F_{4,10} = 3.28$, p=0.109).

Experiment 2: Alongside the above experiment, the same siRNA was used to knockdown *AUTS2* expression during the differentiation process. Results show no significant differences in *AUTS2* expression between non-silencing siRNA and *AUTS2*-targeting siRNA in any of the

stages of differentiation (Figure 34,B). Data is representative of one experiment. An observation that is worth noting is that the presence of lentiviral particles seems to reduce *AUTS2* expression regardless of the insert (non-silencing siRNA and *AUTS2*-targeting siRNA) in all the stages of differentiation. This reduction is similar in both groups demonstrating that *AUTS2*-targeting siRNA does not silence *AUTS2* expression.

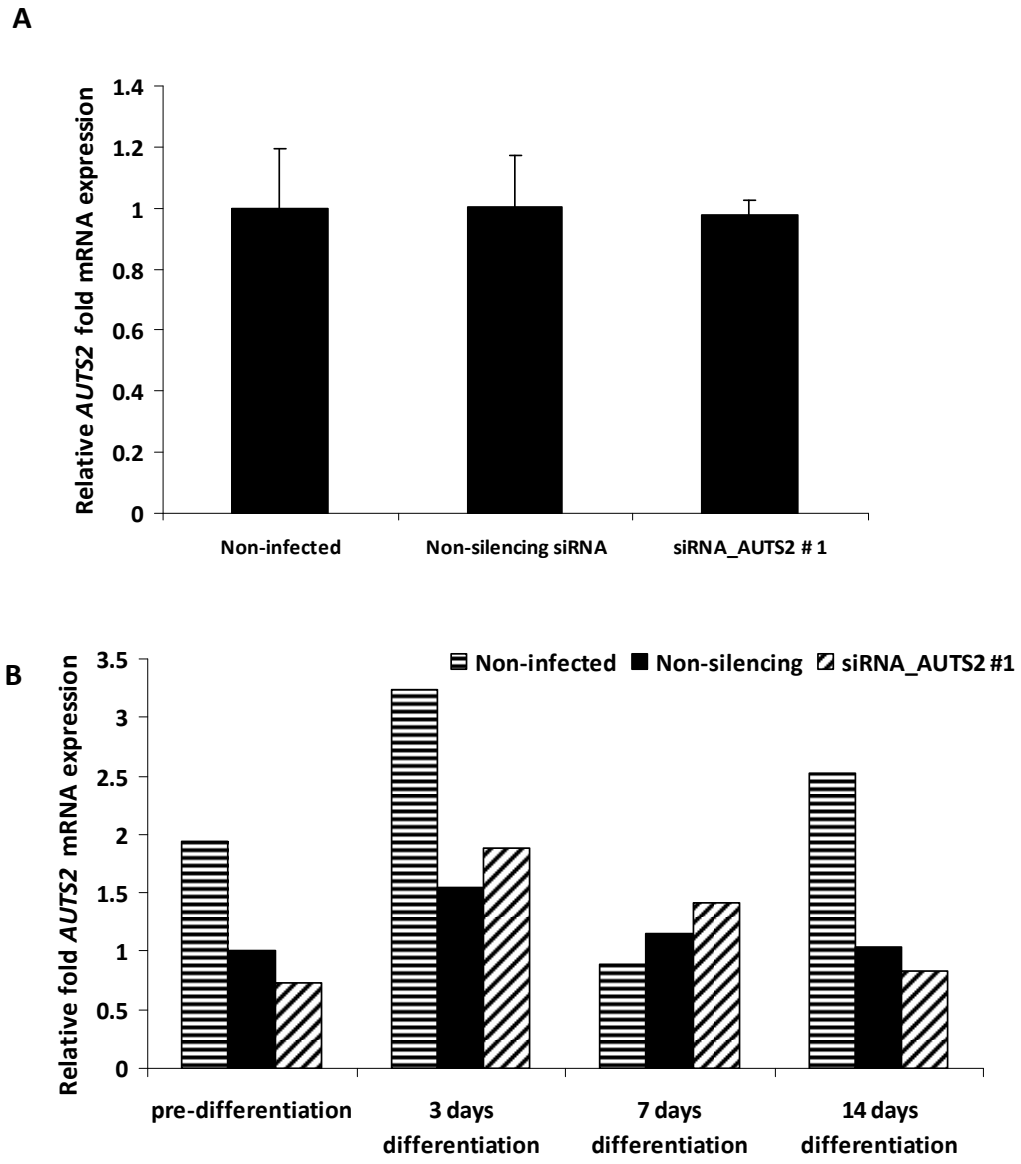
Table 26: Microarray analysis of selected genes involved in neurodevelopment for 7 days SPC-04 differentiation for non-silencing vs siRNA_*AUTS2*.

No significant changes in global gene expression profiles were seen between any groups. No knock down of AUTS2 was detected after 7 days differentiation. Other genes involved in various cellular processes for neurodevelopment are listed below. A Differential Score of +/- 13, is the equivalent of a P value < or = 0.05 where +/- indicates directionality of the differential expression (up or down regulation).

SYMBOL	Non Silencing siRNA		*AUTS2* siRNA		Diff Score	Chr	Description
	Avg Signal	Array StDev	Avg Signal	Array StDev			
AUTS2	4572.7	702.874	4419.1	548.882	-0.001	7	Autism susceptibility candidate 2
CEND1	190.6	10.218	275.4	13.092	0.001	11	Cell cycle exit and neuronal differentiation 1
PAFAH1B1	5492.4	1123.935	5928.8	993.808	0.001	17	Platelet-activating factor acetylhydrolase, isoform Ib
GFAP	12466.7	5542.773	13813.4	8048.107	0.001	17	Glial fibrillary acidic protein
TSC1	813.7	200.529	861.2	240.879	0.001	9	Tuberous sclerosis 1
RUFY3	1094.4	324.047	1239.6	556.044	0	4	RUN and FYVE domain containing 3
NRXN2	24.5	21.579	16.9	10.554	0	11	Neurexin 2 (NRXN2)
NPTX1	32.2	25.929	13.6	21.275	0		PREDICTED: neuronal pentraxin I
SYNGR1	68.3	29.367	87.6	49.353	0	22	Synaptogyrin 1 transcript variant 1b, mRNA.
CDK5R1	1164.2	381.941	1111	226.356	0	17	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)
NRCAM	6514.2	2446	6290.4	1685.686	0	7	Homo sapiens neuronal cell adhesion molecule
GAD1	773.7	72.459	742.1	235.402	0	2	Glutamate decarboxylase 1 (brain, 67kDa), transcript variant GAD25
ARHGEF7	1029.8	59.575	966.2	14.098	0	13	Rho guanine nucleotide exchange factor (GEF) 7, transcript variant 1, mRNA.
COPG2IT1	109.2	53.492	71	11.019	0	7	COPG2 imprinted transcript 1 (non-protein coding)
NES	35.9	25.157	19.2	11,247	0	1	Nestin (NES)
MSI1	89.7	80.787	101.6	60.875	0	12	MSI1 musashi RNA-binding protein 1
SOX1	65.6	39.934	47.6	23.981	0	13	SOX1 SRY (sex determining region Y)-box 1
BASP1	1208.4	1840.204	594.5	705.352	-0.001	5	Brain abundant, membrane attached signal protein 1
INA	3411.4	1140.874	2296.4	641.606	-0.001	10	Internexin neuronal intermediate filament protein, alpha (INA)
SYN1	58.8	49.832	44.5	18.083	-0.001	X	Homo sapiens synapsin I
STMN2	690.1	1179.64	135.5	205.947	-0.001	8	Stathmin-like 2
ERC2	133.5	46.334	95	30.84	-0.001	3	ELKS/RAB6-interacting/CAST family member 2
S100B	17.5	14.944	15.4	12.584	-0.001	21	S100 calcium binding protein, beta (neural) (S100B)

Figure 34: Effect of the *AUTS2*-targeting lentiviral construct on *AUTS2* expression in undifferentiated SPC-04 cell line and upon inducing differentiation.

A) *Undifferentiated SPC-04 cell line was infected with lentiviruses carrying a vector either encoding a sequence that does not target any mammalian sequence (denoted non-silencing siRNA) or encoding a siRNA that previously knocked down AUTS2 expression in 293T cells. Quantification of mRNA levels was performed by real-time qPCR (mean + S.E.M from 3 independent experiments). There was no significant difference between groups p=0.109.* **B)** *The cell line was infected with the abovementioned lentiviruses upon inducing differentiation. Note: error bars are not present as fold expression is calculated from one independent experiment. For both graphs, expression was measured relative to non-silencing siRNA.*



5.5 Summary and Discussion

The results presented above aimed at delving further into the neurodevelopmental role of *AUTS2* using human foetal neural stem cell line SPC-04, to model neural stem cell differentiation. The idea was to silence *AUTS2* expression in a neural stem cell line (undifferentiated and differentiated) and investigate its effect on neural stem cell differentiation, proliferation and cell survival. Additionally, another goal was to identify differential gene expression upon *AUTS2* silencing which would enable the identification of gene networks that interact together with this gene.

A couple of commercially available *AUTS2*-targeting microRNA-adapted shRNAs (siRNAs) were tested for their silencing efficiency initially in 293T, a cell line that endogenously expressed this gene at relatively high levels; its expression was found to be similar to that of the house keeping gene *ACTIN*. As explained earlier, this cell line is easy and less costly to maintain and was thus used to test knockdown efficiency of the siRNAs. qPCR analysis of these cells transduced with one of the siRNA (targeting specifically the medium and long isoforms of *AUTS2*) showed a 70% overall knockdown efficiency (based on three independent experiments).

Thus, this siRNAi was then used to transduce a newly established human foetal neural stem cell line, SPC-04. At the time of writing this thesis. there were no publications on genes that could be knocked down in this cell line. However a PhD student within our group did successfully knock down another gene, Myt1l, in this cell line (unpublished data).

Together, the expression of the medium and long isoforms of *AUTS2* in SPC-04 is slightly lower than that in the 293T cell line; however the expression level is adequate to detect a knockdown. This cell line was transduced at two stages in triplicates: undifferentiated (experiment 1) and upon inducing differentiation i.e. during the addition of differentiation-inducing factors and removal of growth factors (experiment 2). RNA extracts were collected for the following stages of the differentiation process: 1) undifferentiated (only experiment 1); 2) pre-differentiation (upon removal of differentiation-inducing agents; lentiviruses were also removed at this stage); 3) three days differentiation and 4) seven days differentiation for non-infected, non-silencing and *AUTS2*-siRNA. Approx 85-90% transduction efficiency was seen at each of these stages.

There were no distinctive visible differences in morphology or cellular features such as cell appearance, apoptosis, and cell proliferation between cells infected with lentiviruses containing non-silencing siRNA and siRNA targeting *AUTS2* transduced at both stages (undifferentiated and during differentiation). So quantitative analysis assessing these features was not performed.

Microarray analysis was also performed on extracts from both experiments. A surprising finding however, was that despite the visible change in morphology after inducing differentiation where the cells looked elongated, there were no differentially expressed genes detected in the microarray experiments between any of the stages of differentiation tested. It is expected that progenitor cells that undergo differentiation, will have stopped proliferating thus it would be expected that genes responsible for this cellular event would be down regulated. Also, as cells differentiate into more specialised cell types, markers of undifferentiated phenotype such as Sox1, Nestin and Mushahi will decrease while neuronal-, glial- and oligodendrocytic- specific markers will start to increase. The microarray probes were present for these markers but showed no significant changes in the expression of these genes. It is difficult to speculate on the likely cause of this considering that sample preparation for microarray was performed by an external facility. However, it is worthy to mention that working with the SPC-04 cell line had been a challenging task as there were difficulties in inducing differentiation. Furthermore, the biological replicates did not 'behave' similarly i.e. despite treating all replicates similarly, some replicates yielded dying or dead cells. Thus, for the above experiments, six biological replicates were carried out in order to ensure that cell lystes were obtainable upon completion of the experiment. In light of these points raised, it may be that a possible discrepancy for microarray result could be due to two reasons: 1) the stochastic nature of this cell line in which cells randomly 'behave', would obviously result in inconsistencies across the triplicates. 2) In terms of differentiation, the triplicates may contain different proportions of neurons, astrocytes and oligodendrocytes. In fact, a mixture of cell types is thought to be a major source of biological variation in microarray experiments (Imbeaud & Auffray, 2005). Additionally, it has been shown that even if 'genetically identical cells' are cultured under 'identical' conditions, there is considerable disparity in gene expression profiles between them (Blake *et al.*, 2003). Early passage cells were used for the above experiments, to circumvent this issue.

In retrospect, since this cell line had not been studied previously and is not yet available commercially, it would have been advisable to investigate the characteristics of this cell line before conducting such an experiment. For example, carry out initial immunostaining analysis to look at number of each cell type in order to evaluate its consistencies in differentiation.
One reason why there was no significant differential gene expression detected in the data set, is because the there were large standard deviation between arrays which could arise from either an abnormal biological sample, RNA preparation or poor hybridisation. Removal of potential outliers from the data set during analysis would decrease the standard deviations and possible increase the chances of finding significant changes in gene expression.

An equally disappointing finding from the microarray data and subsequent qPCR analysis was that there was no significant difference in *AUTS2* expression between those infected with non-silencing siRNA and with siRNA targeting *AUTS2* neither in undifferentiated nor differentiated SPC-04. The microarray contained one probe for *AUTS2* which detected the long and medium isoform. Likewise, amplification of the same isoforms was detected by primers for qPCR. The lack of *AUTS2* knockdown in the SPC-04 is surprising as this siRNA clearly knocked down the medium and long isoforms in the 293T cell line. One possible explanation for the discrepancy between the two cell lines could be that perhaps the intrinsic silencing mechanisms may differ. While in 293T cells, the siRNA silencing mechanism may involve the degradation of RNA, it is possible that in the SPC-04 cell line the mRNA may be prevented from being translated. In order to corroborate this idea, protein lysates from the above experiments are required to carry out Western Blot using the anti-AUTS2 antibody that was previously used in Chapter 4. Due to time constraints to finish writing this thesis, this was not performed. Protein lysates were not collected for the above experiments so the whole experiment would need to be repeated. Nonetheless, an alternative cell line, preferably of neuronal origin could have been used to test for knockdown efficiency of AUTS2. The human neuroblastoma cell line SH-SY5Y was available in the laboratory but it expressed AUTS2 only at low levels. In hindsight we should have tested the siRNA in the SPC-04 cell line itself before performing microarray analysis. Ultimately, it is important to verify that AUTS2 is silenced at the protein level. Thus, the antibody characterised in the previous chapter could be used to corroborate this in the 293T as well as the SPC-04 cell lines.

In terms of future studies, further research is required to determine the role of *AUTS2* in neurodevelopment. Ideally, more siRNAs will require to be tested in this specific cell line

before experimentation. In the Chapter 3.4, shRNA oligos were manually designed according to Hannon's design strategy (Paddison *et al.*, 2004). Although it is a more time consuming process to produce these shRNA constructs, were demonstrated to efficiently silence their targets *in vitro*. Furthermore, the antibody characterised in the previous chapter would confirm the knock down of AUTS2 at the protein levels which is ultimately what we want.
As the SPC-04 cell line is not a commercially available cell line, perhaps more optimisation is required prior to its application to research experiments. Alternatively other neural stem cell lines could be used; for example human embryonic stem cells lines have been efficiently used as an *in vitro* model of neurodevelopment in order to study gene function (Shin *et al.*, 2006; Kirkeby *et al.*, 2012; Tirotta *et al.*, 2012).

In conclusion, neural stem cells are good models for neurodevelopment especially for their ability to manipulate gene expression at various stages of differentiation in order to investigate gene function. Although, the SPC-04 cell line used in this chapter was used without success to ascertain the neurodevelopmental role of *AUTS2 in vitro*, further work is required to achieve this using different neural stem cell lines and/or different siRNAs.

Chapter 6 Discussion

6.1 Summary and discussion of data chapters

The general aim of the work reported in this thesis was to investigate the functional role of three genes involved in neuropsychiatric disorders, dopamine receptors D2 and D3 as well as AUTS2 using lentiviral mediated gene delivery as a primary method of gene transfer.

The first data chapter aimed to establish the above technique in vitro and in vivo using two dopamine receptor genes encoding for dopamine D2 and D3 receptors (D2R and D3R), for which their roles in locomotor activity has been well characterised in the literature. Lentiviral-mediated gene knockdown and overexpression constructs for these genes were developed to specifically manipulate expression levels of these genes in the rat NAcc, a brain area important for spontaneous and induced locomotor responses. The findings from this chapter first showed that the lentiviruses generated indeed overexpressed and silenced D2R and D3R in vitro. Furthermore, the results demonstrated that NAcc D2R and D3R share a similar function in spontaneous locomotor activity in a novel environment though no contribution of these receptors to locomotor activity in a familiar environment was observed. However, there was a difference in their role in visual stimulation-induced locomotor activity. While NAcc D2R seems to limit light-induced activity, as silencing NAcc D2R potentiated this behaviour. On the other hand, NAcc D3R slightly reduced light-induced activity. Immunohistochemical staining of the brain sections from the different groups demonstrated that the injections were indeed localised in the NAcc and that the lentiviruses targeted specifically neuronal cells in this region. In order to completely validate this technique, *in vivo* confirmation of overexpression and knockdown of both D2R and D3R, either at the RNA or protein level, is required.

The next chapter specifically aimed at determining the sub-cellular localisation of AUTS2 and its interacting protein partners. Thus, to achieve this assessment of a commercially available anti-AUTS2 antibody was carried out to verify its target specificity. This was done by creating a lentiviral plasmid overexpressing AUTS2 either in its native form or as a fusion protein to give V5-tagged AUTS2. Using anti-AUTS2 and anti-V5 antibodies, western blot analysis of 293T lysates transfected with the above constructs revealed that AUTS2 was indeed overexpressed at the protein level. Immunocytochemical analysis using the anti-AUTS2 antibody, on Neuro2a, a mouse neuroblastoma cell line showed that this protein was endogenously localised in the nucleus. This finding was confirmed by immunocytochemical analysis using the anti-V5 antibody on lysates overexpressing V5-tagged AUTS2 in the 293T cell line. These findings

confirm a previous report by Bedogni et al. (2009) in which immunostaining was performed on mouse brain slices using a different anti-AUTS2 antibody. Interestingly, in this paper, AUTS2 was specifically localised in neuronal nuclei. Data from this chapter has not only confirmed AUTS2 as a nuclear protein but also has validated a commercially available antibody that can be used for Western Blot and immunostaining for future studies.

Next, having verified that the overexpression vector constructs and antibodies worked, both antibodies were used to co-immunoprecipitate AUTS2 in order to 'pull out' its potential binding/interacting protein partners. However, these antibodies were found to be unsuitable for co-IP despite a number of optimisations of the technique. In order to obtain some indication of its function, in silico analysis for AUTS2 had limited information on its potential interacting protein partners with little data from experimental evidence. However, the recent finding that AUTS2 and AUTS2-related proteins were recovered through tandem affinity purification of PCGF proteins (Gao et al., 2012), which are involved in suppression of gene expression by modification of histone protein during embryonic development, suggest that it may be part of a protein complex and is important early in development. As discussed earlier, the yeast two-hybrid system would enable the identification of other protein interacting partners and confirm its association with PCGF proteins. Clearly further investigation is required to verify and extend upon the above findings.

Given its implications in a broad range of psychiatric disorders and its characterisation as a neurodevelopmental gene (Sultana et al., 2002; Richler et al., 2006; Bakkaloglu et al., 2008; Hamshere et al., 2009; Elia et al., 2010; Komoike et al., 2010; Mefford et al., 2010; Schumann et al., 2011), very little is known about the functional role of *AUTS2* in neurodevelopment. Chapter 5 attempts to investigate this using lentiviral-mediated gene silencing of *AUTS2* in human neural stem cell line, SPC-04. For this purpose, a couple of commercially available lentiviral plasmids containing microRNA-adapted shRNA (siRNA) (Boden et al., 2004; Siolas et al., 2005) targeting *AUTS2* were obtained. Their target silencing efficiency was assessed in the 293T, human embryonic kidney cell line which are easy to culture, less costly to maintain and endogenous *AUTS2* expression was detected at relatively high levels. qPCR analysis of RNA extracts from cells transduced with one of the siRNAs showed a 70% overall knockdown efficiency (based on three independent experiments). This siRNA was then used to transduce the newly established human foetal neural stem cell line, SPC-04 at two stages of differentiation: undifferentiated and during differentiation. At the morphological level, there

were no visible differences between cells infected with AUTS2-targeting siRNA compared to control (non-silencing siRNA).

A previous microarray analysis conducted prior to our experiments, (performed by Dr Desrivieres) in which the gene expression profile was looked at in undifferentiated, predifferentiated, 3 days differentiation and 7 days differentiation revealed a number of genes involved in synaptogenesis and axonogenesis such as *SNAP25, SYN1, NRXN2, INA, ELMO1* were positively correlated with *AUTS2* expression across all the differentiation stages analysed (unpublished data) although a batch effect for found in the triplicates. The plan was to repeat the experiment without obtaining a batch effect by performing the triplicates simultaneously and the idea was to see how the expression of these genes would change if *AUTS2* expression was silenced. Microarray analysis was carried out at the undifferentiated stage, during differentiation, 3 days and 7 days differentiation. Disappointingly, analysis of *AUTS2* mRNA expression specifically the medium and long isoforms which are targeted by the siRNA showed no *AUTS2* knockdown effect in any of the stages of differentiation and this finding was confirmed by qPCR analysis. Even more surprising was the finding that microarray analysis showed no significant changes in global gene expression profile between any of the differentiation stages despite the morphological changes observed during the differentiation process. As discussed earlier this could be due to heterogeneous cell population even within the biological replicates resulting in variation in gene expression profiles. These incongruities were found in a similar experiment conducted by another student in our group. qPCR results confirmed that the siRNA did not knockdown *AUTS2* in this cell line. In hindsight, before conducting microarray experiments, it would have been advisable to analyse *AUTS2* expression to verify knockdown in this cell line even if this had already been done in a different cell line.

Although the above chapters aimed at finding out the role of AUTS2 in neural function, clearly further research is required. Based on publically available online data for human and rodent *AUTS2* gene expression, a few interesting comments, on the possible function of *AUTS2* during development have been outlined below. Of course information obtained from these resources have to be interpreted with some caution.

Looking at rodent gene expression in GenePaint which is a well established collection of *in situ* hybridisation experiments of the mouse developing brain for E10, E14.5, E15.5, post natal stages P7 and P56. This database showed that *Auts2* is highly expressed in the developing

neocortex of the mouse brain. Although it would be very useful to look at *Auts2* expression at all these different stages, unfortunately, data for *Auts2* expression is only available for E14.5. Nonetheless, this data shows that *Auts2* is relatively highly expressed in the developing neocortex in agreement with previous in situ hybridisation findings (Bedogni et al., 2010b). Additionally, these results show that T-box brain 1 (*Tbr1*) is highly expressed in the same region which is not surprising as it transcriptionally activates neocortical *Auts2* expression (Bedogni et al., 2010a). Given that several lines of evidence associate *Tbr1* with neocortical differentiation, it is probable that *Auts2* may also be involved in this process. For example, *Tbr1* was shown to be important for the transition of intermediate progenitors to post mitotic neurons in the neocortex (Englund et al., 2005; Bedogni et al., 2010a). Furthermore, Tbr1 mutant mice display profound defects in frontal differentiation, and *Auts2* expression was also reduced along with two other genes implicated in brain development *Bcl2, Rorb* and *Reln* (Reelin), a gene known to regulate neuronal migration and positioning in the brain.

The neocortex is a brain region involved in higher brain functions such as motor commands, sensory perception, spatial reasoning, regulating thought and emotional processes and language. It has been shown that aberrant neocortical organization and circuitry may result in cognitive dysfunction and increase propensity to neuropsychiatric disorders (Valiente & Marin, 2010; Rubenstein, 2011). It is now well known that the cognitive abilities unique to humans are due the large size of the neocortex compared to other species. In rodents the neocortex is smooth while in primates, it contains folds and grooves (sulci and gyri) to increase surface area and which has evolved to perform higher brain functions.

Despite the differences in structural features and cognitive abilities of the rodent and human brain, analysis of the human brain transcriptome was carried out. This data, obtained from the Human Brain transcriptome database (Kang et al., 2011) contains gene expression profiles associated with neurodevelopmental processes such as synaptogenesis, dendrite development, cell proliferation and axonogenesis. In addition, it contains gene expression trajectories of specific genes in different human brain regions before and after birth specifically ranging from developmental stage post-conception week 5.7 (when the neural tube starts to form) through to 82 years of age. Data is generated from 57 developing and adult post-mortem brains from males and females of multiple ethnicities. Expression profiles are shown for six different brain regions (neocortex, striatum, hippocampus, mediodorsal nucleus of the thalamus, amygdala, cerebellar cortex) starting from 4 weeks post conception until 82 years of age. The data generated showed that AUTS2 is expressed post and pre-natally

(Figure S2, supplementary materials). Despite the small sample size, the various brain regions analysed such as neocortex, hippocampus, striatum, mediodorsal nucleus of the thalamus, cerebellar cortex all shared a similar expression patterns for AUTS2. Interestingly, its expression peaks briefly during 10-13 week post conception, a period when cell proliferation and migration are key cellular processes. Furthermore, this database contains information on general gene expression profiles of co-expressed genes involved in specific biological processes such as cell proliferation, synaptogenesis, axon formation and myelination with distinct spatio-temporal expression patterns are provided in the database. A systematic search to see if *AUTS2* expression pattern matched that of any of these modules was performed. Intriguingly, the expression pattern of *AUTS2* resembles that of DCX, doublecortin, a gene important for neural migration but also proliferation and differentiation. In light of the above findings, it is tempting to speculate that *AUTS2* may be involved in these cellular processes. Further support for this view, comes from the observation that *Auts2* is highly expressed in the subventicular zone, the area of the developing brain in which neurons are generated and from which they migrate (Bedogni et al., 2010a). Very recently, it has been shown that *AUTS2* silencing in zebrafish using morpholinos (antisense technology), resulted in small head size, reduced neuronal number and locomotor dysfunction (Oksenberg *et al.*, 2013) further supporting its role in neurodevelopment and brain function. Interestingly, PCGF3, one of the proteins with which AUTS2 was isolated using Tandem Affinity Purification as described in Chapter 4, also shared a similar expression trajectory of AUTS2 (see Figure S3 in supplementary materials), suggesting it may interact with this protein to execute its function. However, this is only speculation based on the above data; they only give clues that point to AUTS2 function. *In vitro* and *in vivo* experimental evidence is clearly required for validation of the above information.

6.2 Project limitations and future directions

Limitations specific to each study has been discussed in detail in the relevant sections. There are some limitations to this project as a whole that should be noted. Here, a discussion is presented on what could have been done differently if the project was to be done again. An issue that concerns the thesis as a whole, is producing accurate gene quantitation by properly normalising the qPCR data. Throughout this thesis, only one internal control gene has been used. There is growing evidence that normalising to a single housekeeping gene can introduce

large and variable errors in the analysis as the expression of these genes vary depending on the tissue types, experimental conditions and time points. Thus it is important to ensure reliable and accurate results, normalising based on multiple reference genes is required (Vandesompele *et al.*, 2002). GeneNorm is a commonly used software that uses an algorithm to determine the most stable reference gene from a group of tested candidate housekeeping genes in a particular sample (Vazquez-Blomquist *et al.*, 2012). If I had to repeat the projects in this thesis, I would use Genorm or a similar algorithm-operated software to normalise qPCR data.

In Chapters 4 and 5, where AUTS2 cellular and molecular functional characterisation was sought, there are number of things I would have done differently. I would design shRNAs targeting human *AUTS2* as I did for D2R and D3R. Ideally the shRNA would target all *AUTS2* three isoforms so that there would be no compensatory mechanisms, for example, increase in expression of the short isoform to compensate for the lack of expression of its longer transcript counterparts. In terms of *in vitro* experiments, the shRNA designed to knockdown the three isoforms of AUTS2 can be tested for its silencing efficiency (at RNA as well as protein level using the antibody characterised in Chapter 4) in a neural cell line that expresses this gene at high levels. For example, commercially available human neural cell lines can be used to not only test if the shRNAs silence *AUTS2* but also investigate the consequence of various cellular processes such as cell survival, proliferation and differentiation on these cells. Human embryonic stem cell- and feotal- derived neural progenitors have been used to investigate neural proliferation, differentiation and even migration *in vitro* previously (Delaloy et al., 2010; Li et al., 2012). For example, ReNcell VM (Millipore, UK) is an immortalized human neural progenitor cell line with the ability to readily differentiate into neurons and glial cells. ReNcell VM was derived from the ventral mesencephalon region of human fetal brain which has been immortalized by retroviral transduction with the v-myc oncogene (Liedmann *et al.*, 2012). Several publications have used this cell line to investigate differentiation potential upon specific gene manipulation (Hubner *et al.*, 2010; Lange *et al.*, 2011). I would carry out immunostaining in undifferentiated cells such as Sox1 and Nestin as well as differentiation markers such Tuj1 (neurons) and GFAP (glia) to investigate the differentiation potential in this cell line and to determine the time period required for producing mature neurons and glial cells.

Additionally, I would look for AUTS2 expression level (during various stages of differentiation) using the antibody I validated in Chapter 4. In the case where AUTS2 is expressed at a specific stage in differentiation, I would test for the silencing efficiency of AUTS2-targeting siRNA that I designed as explained above. If the siRNA significantly silenced AUTS2, I would then go on to investigate its effect on differentiation using the abovementioned differentiation markers. I would also conduct proliferation assays using BrdU assays for example. In the case that AUTS2 is expressed in all stages of differentiation, I would systematically knock down AUTS2 at each stage and investigate the abovementioned cellular processes. Furthermore, co-expression analysis as outlined in Chapter 5 can be performed to investigate expression profiles of genes involved in neurogenesis and brain development in order to determine a possible molecular pathway through which *AUTS2* may exert its function.

Ultimately, *in vivo* findings would further our understanding of the contribution of this gene in mediating behavioural traits. Thus, manipulation of *Auts2* expression in the brain will enable us to investigate the consequence of its absence in the developing brain on behaviour in mice. The Knockout Mouse project (KOMP) repository is an initiative to generate null mutation mice for every gene in the genome. Reference to its database showed that a knockout mice for *Auts2* is not yet available. Indeed, knockout mice can be generated using lentiviral mediated gene delivery (Singer *et al.*, 2007). It would be interesting to not only perform a battery of behavioural paradigms on these mice in order to study their cognitive, emotional and other behavioural function compared to their wild type counterparts. It would also be interesting to compare differences in brain sizes, overall brain architechture, cell proliferation, migration and differentiation between mutant and wild type mice both pre-natally and post-natally. Findings from this would indeed further our understanding on the contribution of AUTS2 in brain function and development.

6.3 Concluding remarks

The pathways leading from a specific gene to behaviour are complex. It is important to genetically dissect the molecular mechanisms which mediate behavioural traits in order to understand the underlyinging basis of neuropsychiatric disorders. To sum up, using dopamine receptors, this thesis has shown how lentiviral mediated gene delivery *in vitro* and *in vivo* can be used to delineate the role of different gene isoforms in distinct brain regions to investigate behaviour. The present series of findings also aimed to functionally characterise *AUTS2*and have highlighted the need to further investigate its importance in brain function and behaviour. It is hoped that the findings presented here may form the basis for future studies aimed at further elucidating the functional role of candidate genes such as *AUTS2*, and thus increasing our understanding of the molecular underpinnings of neuropsychiatric disorders in general.

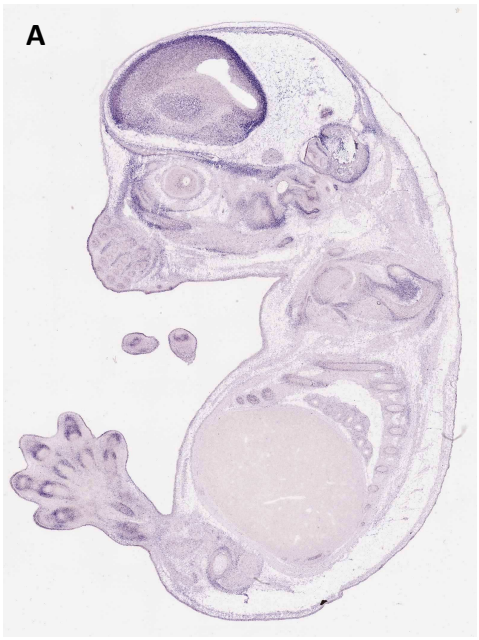
Supplementary materials

Figure S1: Relatively high neocortical expression of *Auts2* in the developing mouse

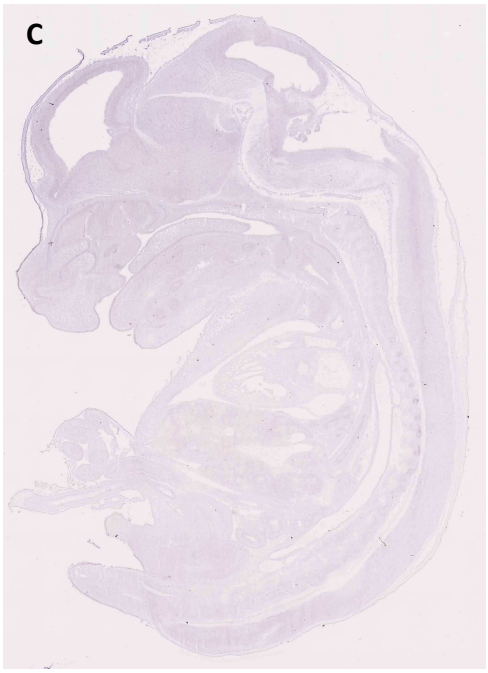
In situ hybridisation at mouse embryonic stage 14.5 obtained from GenePaint (Visel *et al.*, 2004), www.GenePaint.org which contains gene expression patterns on tissue sections by RNA *in situ* hybridisation at mouse embryonic stages E10.5, E14.5, E15.5 and post-birth stages P7 and P56. This figure shows that mRNA expression of *Auts2* is present in several tissues such as the cartilage of limbs and soft tissues of the nasal cavity in the mouse embryo but comparatively high expression is seen in the developing brain especially the neocortex as well as the peri-ventricular zone of the neocortex and the developing cortical region.

Figure S1: *In situ* hybridisation analysis of the mouse embryo E14.5 generated from GenePaint database (Visel *et al.*, 2004) for A) *Auts2*; **B)** *Tbr1;* **C)** *Drd3.*

For reference, the developing tissues of the embryo are labelled in **D.** *Auts2 is expressed in the developing mouse brain, specifically the progenitor neurons of the neocortex. Tbr1 is highly and specifically expressed in the developing neocortical region while Auts2 is moderately expressed in the same region and is also detected in other regions such as the cartilage in the limbs. For comparison, Dopamine receptor Drd3 expression is low at this stage of development.*


A


B


C

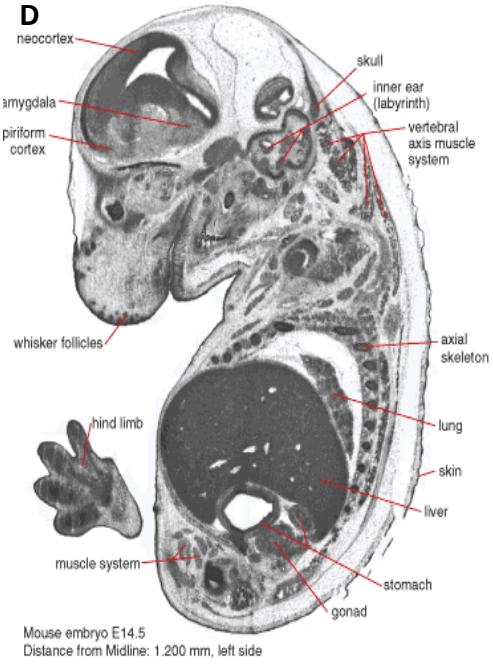
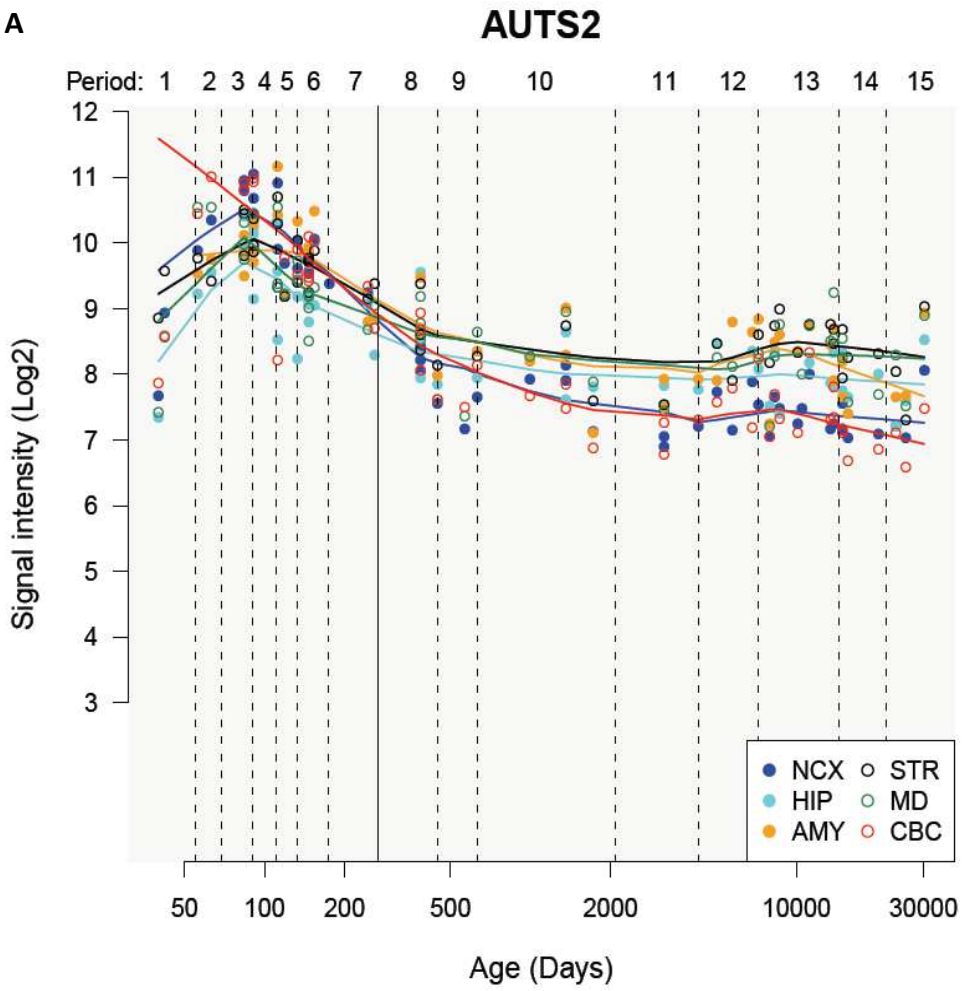
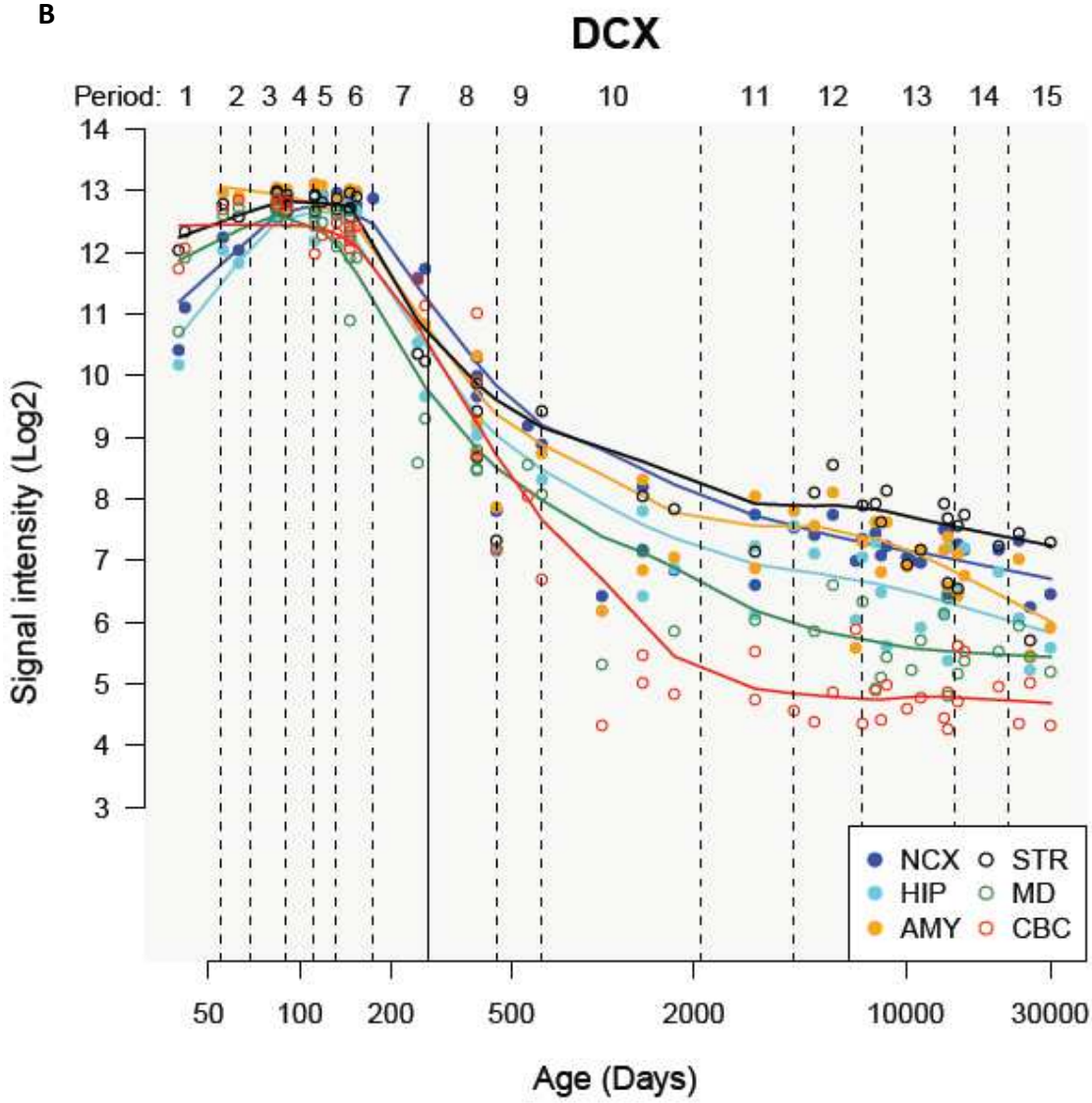

D
neocortex
amygdala
piriform cortex
whisker follicles
skull
inner ear (labyrinth)
vertebral axis muscle system
axial skeleton
lung
skin
liver
stomach
gonad
hind limb
muscle system
Mouse embryo E14.5
Distance from Midline: 1.200 mm, left side

Figure S2: Expression trajectories of A) *AUTS2* B) *DCX* in the developmental stages of the human brain.

These figures are generated from 'The Human brain transcriptome' database. The solid vertical line between period 7 and 8 indicates birth. The developmental stages range from post conception week 5.2 to 83 years of age. DCX is doublecortin which is a microtubule-associated protein vital for neuronal migration. NCX: Neocortex; STR: Striatum ;HIP: Hippocampus; MD: Mediodorsal nucleus of the thalamus AMY: Amygdala; CBC: Cerebellar cortex



B



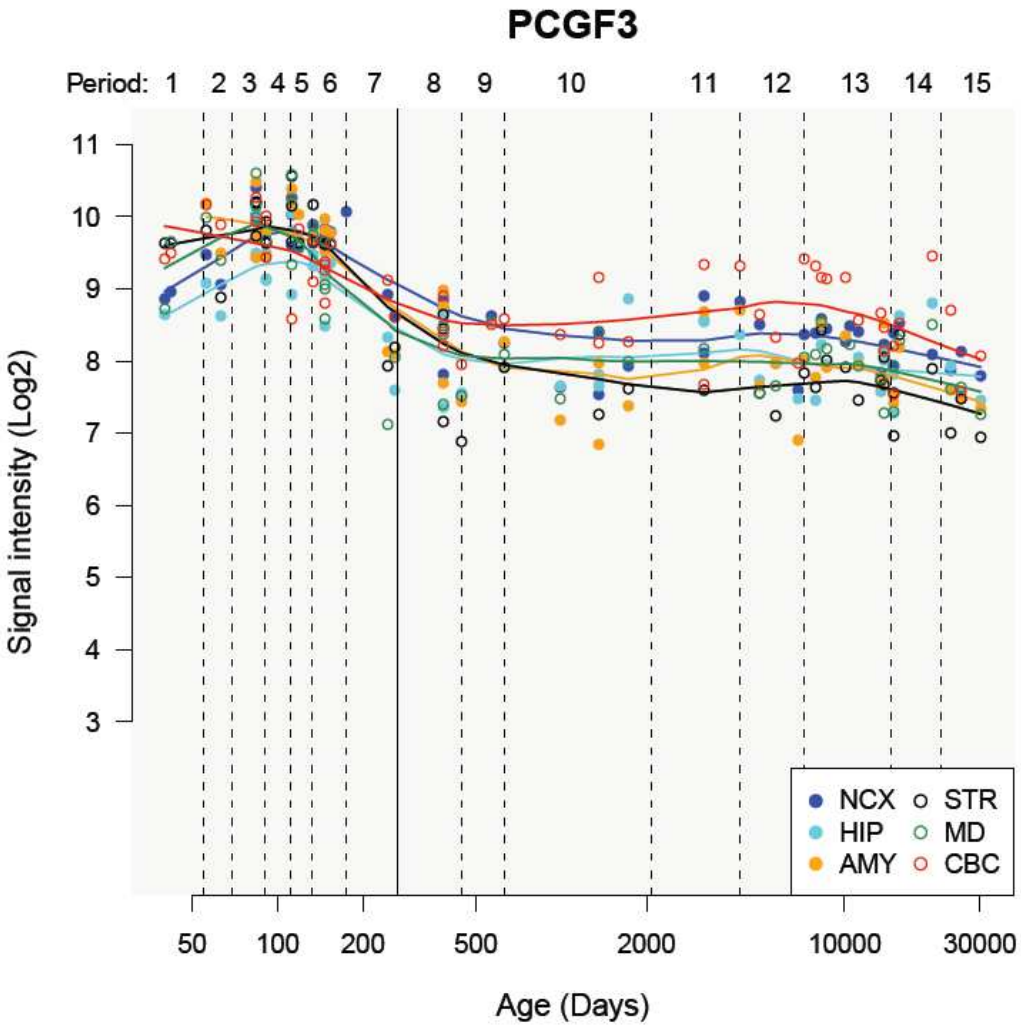
Human *AUTS2* is expressed at its peak when proliferation and migration are key processes

The Human Brain Transcriptome Database (**http://hbatlas.org (Kang *et al.*, 2011))** gene expression profiles associated with neurodevelopmental processes such as synaptogenesis, dendrite development, cell proliferation and axonogenesis. In addition, it contains gene expression trajectories of specific genes in different human brain regions before and after birth. Data is generated from 57 developing and adult post-mortem brains from males and females of multiple ethnicities. A systematic search was carried out to compare AUTS2 expression profile with other genes prominent in various cellular processes important in brain

development in order to pick out any correlation that may point out to its role in development. Figure S2, (A) depicts the expression trajectory of *AUTS2* in 6 different brain regions starting from 5.7 weeks post conception until 82 years of age generated from 57 developing and adult post-mortem. The brain regions analysed are: neocortex (NCX); striatum (STR); hippocampus (HIP); mediodorsal nucleus of the thalamus (MD); amygdala (AMY); cerebellar cortex (CBC). It can be seen that generally, *AUTS2* is highly expressed in the human brain during the lifetime although its expression is higher in the prenatal stage of the developing brain compared to postnatally. Its expression peaks at period 3 (between 10 and 13 weeks post conception) representing the early foetal development period, and gradually decreases after birth until it stabilises at the end of period 12 corresponding to adolescence. Also evident is a generally small rise in the expression pattern in young adulthood (period 13, represented between 20-40 years of age). Interestingly, the overall expression pattern of *AUTS2* closely matches that of a neuronal migration marker doublecortin, *DCX* (Figure S2,B).

Figure S3: **Expression trajectory of *PCGF3* in the developmental stages of the human brain.**

This gene is involved in the regulation of embryonic development although its mechanisms are unknown. Recently, AUTS2 was found to interact with the protein through tandem affinity purification (see Chapter 3.1). Interestingly, its expression trajectory is similar to that of AUTS2 (refer to Chapter 3.2 for further details).



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